Identification of the MNN2 and MNN5 Mannosyltransferases Required for Forming and Extending the Mannose Branches of the Outer Chain Mannans of Saccharomyces cerevisiae*

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The mannan structure found on the N-linked glycan of the yeast Saccharomyces cerevisiae is composed of a long backbone of α-1,6-linked mannose to which are attached branches consisting of two α-1,2-linked mannoses followed by an α-1,3-linked mannose. In the mutants mnn2 and mnn5, the addition of the first and second of these two mannoses, respectively, is defective. In this paper, we report the identification of the genes corresponding to these mutations. The two genes encode closely related proteins with distant homology to the known Mnn1p α-1,3-mannosyltransferase. We show that these proteins are localized in an early compartment of the yeast Golgi and that they are not physically associated with each other or with the two protein complexes known to be involved in synthesizing the α-1,6-linked backbone. The identification of Mnn2p and Mnn5p allows us to assign Golgi proteins to all of the catalytic steps in S. cerevisiae mannan synthesis.

The N-linked oligosaccharides on the glycoproteins of the yeast Saccharomyces cerevisiae exist in two major forms, which differ in the length of a mannan outer chain attached to the N-linked core (1, 2). The glycoproteins of the internal compartments have a single α-1,6-linked mannan attached to one arm of the core, to which two or three further mannoses may be added (3, 4). In contrast, many of the proteins of the cell wall and periplasmic space have attached at the same position a large “mannan” structure of up to 200 mannoses. This consists of a long α-1,6-linked backbone with branches of up to three residues in length, the first two being α-1,2-linked and the final one being α-1,3-linked (Fig. 1A) (1, 3, 5). In addition, a phosphomannose residue is attached to the first mannan on some of the branches. The large amount of mannan in this structure results in mannoproteins being 40% of the dry weight of the cell wall (6). These mannans are thought to shield the cell from digestive enzymes and neutralizing antibodies, to contribute to cell wall integrity, and to serve as ligands for cell-cell associations such as flocculation and host cell attachment during invasive growth (6–8). For the soluble proteins of the periplasmic space, the large sugar structures may prevent loss through the cell wall by contributing to hydrodynamic volume. Similar large mannose-containing oligosaccharides are also found on the cell wall proteins of many fungi and yeasts including Aspergillus, Candida, Fusarium, Schizosaccharomyces, and Trichophyton, suggesting that they are a general feature of yeast and fungi (9–13). The backbone is usually α-1,6-linked mannan, and the first branch is usually an α-1,2-linked mannan, but the rest of the side chain varies greatly between species. Mannoses of varying number can be present in a variety of linkages, and other sugars can be incorporated including N-acetylglucosamine and galactose. This variation of the side chain may reflect evolutionary pressures to evade cell wall-digesting hydrolases, immune responses, and sugar-binding killer toxins from other yeasts.

As well as having its own biological relevance, the synthesis of yeast mannan provides as a model system to study how cells make the large and complex carbohydrate structures found in many other species such as the glycosaminylglycans of animals and the hemicellulloses and pectins of plants. Like yeast mannans, these molecules have a long backbone carrying diverse modifications and are synthesized in the secretory pathway rather than being directly extruded through the plasma membrane. Understanding of the mechanism of S. cerevisiae mannan synthesis has come from both genetic and biochemical studies. Conditional mutations that block the yeast secretory pathway have allowed the delineation of the processing steps into the different compartments of the pathway (14, 15). In addition, several genetic screens have identified yeast mutations that cause defects in the synthesis of mannan including the mnn (mannan), och (outer chain), ngd (N-glycosylation defective), and ldh (low dye binding) sets of mutants (16–19). Finally, it has been possible to identify mannosyltransferase activities in yeast extracts and, in some cases, correlate these to the products of genes identified in the above screens (20, 21). From these studies, a basic pathway has emerged. The core structure of the N-linked glycan is attached to nascent proteins in the ER1 and, as in mammals, is trimmed to ManαGlcNAc2 before the proteins leave the ER (22). Upon arrival in the Golgi, a single α-1,6-mannose is initially added to all of the core structures by the Och1p transferase (17, 23). Then, on a subset of proteins, this mannan is extended to a long α-1,6-mannose backbone by two complexes of proteins. The first contains Mnn9p and Van1p, and the second contains Mnn9p, Anp1p, Hoc1p, and Mnn11p (24). At present, it is not clear if all these proteins are α-1,6-mannosyltransferases, but both complexes possess such activity. What causes the Mnn9p-Van1p complex to only initiate this backbone on a subset of yeast glycoproteins is not understood, but it presumably reflects recognition of some feature of the proteins themselves.

Of the enzymes involved in synthesizing the mannan branches, that responsible for adding the final α-1,3-linked mannan has been identified as the product of the MNN1 gene (20, 25, 26). Some of the branches also receive phosphomannan

1 The abbreviations used are: ER, endoplasmic reticulum; ORF, open reading frame; HA, hemagglutinin; TEV, tobacco etch virus; Endo H, endoglycosidase H; PKC, protein kinase C.
groups, and there is evidence that the MNN6 gene product is responsible for this addition (27). In contrast, the enzymes responsible for attaching the two first α-1,2-linked mannoses of the branches are at present unknown. However, two of the mnn mutations specifically affect these steps. Thus, the mnn2 mutant is defective in the addition of the first α-1,2-linked mannose as it has no branches on the backbone (Fig. 1A). Likewise, the mnn5 mutant is defective in the addition of the second mannose as the branches on its mannan consist of a single α-1,2-linked mannose. In this paper, we report the identification of the MNN2 and MNN5 genes and show that they encode proteins that are related to the Mnn1p mannosyltransferase and that are located in the early Golgi of yeast.

EXPERIMENTAL PROCEDURES

Yeast Strains and Plasmids—Standard protocols were used for the growth and mating of yeast strains, transformation by the lithium acetate method, and preparation of genomic DNA and alcaline blue staining (16, 28). The wild-type strain used was SEY6210 (MATa ura3-52 his3Δ200 leu2-3, 112 trp1-901 lys2-801 suck2-3A) (29). Mnn strains LB1–3B (MATa mnn2-1) and LB65–5D (MATa, mnn5) and parental strain X2180 were obtained from the ATCC (30, 31). YBR105c and YBR110c were either disrupted by replacing the entire ORF, or epitope tagged precisely at the N terminus using homologous recombination with appropriate polymerase chain reaction products containing the Schizosaccharomyces pombe HIS3 or Kluyveromyces lactis URA3 genes; polymerase chain reaction was used to check the integrants (32, 33). Epitope-tagging integrations added the amino acid sequence GAGA followed by either three copies of the hemagglutinin (HA) tag or nine copies of the Myc tag. For COOH-terminal protein A fusions, polymerase chain reaction products were amplified from plasmid pZIP-HIS5, which contains the HIS5 marker and encodes a linker sequence starting GAGAGAGAGAGAAGADNVK…, where SENLYFQ/G is the TEV protease cleavage site (34) and VDKN is the start of the first of two synthetic protein A “E” domains (35). Myc-tagged invertase, Mnn1p, Mnt1p were expressed in strains using an integration plasmid or CEN plasmid, respectively (28, 36).

Indirect Immunofluorescence and Immunoblotting—The Myc epitope tag was detected with mouse monoclonal 9E10; the HA tag was detected with mouse monoclonal 12CA5 or rat monoclonal 3F10 (Boehringer Mannheim). In addition, rabbit antisera against both tags (Santa Cruz), Anip1 (24) and α-1,6 mannose (37), were used for protein blotting and immunofluorescence. Total yeast proteins were prepared by resuspending cells at 1 A600 unit per 20 μl of SDS sample buffer, vortexing with glass beads (425–600 μm, Sigma) for 5 min at 4 °C, and denaturing for 5 min at 65 °C. For immunoblotting, proteins were separated by SDS-polyacrylamide gel electrophoresis and then electrophoresed onto nitrocellulose (0.45 μm, Schleicher & Schuell) and probed with antibodies in phosphate-buffered saline, 2% dried milk. Peroxidase-conjugated anti-rabbit and anti-mouse secondary antibodies were detected by chemiluminescence (ECL, Amersham Pharmacia Biotech). Direct immunofluorescence was performed as described previously (38) using fluorescein isothiocyanate-conjugated donkey anti-rat Ig (Southern Biotech, 1/50) and Cy3-conjugated goat anti-rabbit IgG (Amersham Pharmacia Biotech, 1/700) and a Bio-Rad MRC-600 confocal laser-scanning microscope.

Immunoprecipitation and Protease Release—Yeast cell growth was stopped in log phase by addition of 10 mM sodium azide, and 50 A600 units of cells were resuspended in 5 ml of 100 mM Tris-HCl, 10 mM dithiothreitol, pH 9.4, 10 mM sodium azide and incubated at 30 °C for 10 min. Cells were then resuspended in 1 ml of a spheroplasting solution (1 M sorbitol, 50 mM Tris-HCl, pH 7.4, 2 mM MgCl2, 10 mM dithiothreitol, 10 mM sodium azide, 100 units of zymolase (20T, ICN)) and incubated at 30 °C for 30 min, or until spheroplasting was complete. Spheroplasts were then washed in spheroplasting solution without zymolase at 4 °C and lysed at 4 °C for 30 min by gentle rolling in 1 ml of TDI buffer (10 mM triethanolamine, pH 7.5, 150 mM NaCl, 1% digitonin, 2 mM EDTA supplemented by protease inhibitors). Cell debris was removed by centrifugation at 10,000 × g for 10 min at 4 °C. The supernatant was brought to 4 °C with 10 μl of protein A-Sepharose (Amersham Pharmacia Biotech) for 12CA5 immunoprecipitations or protein G-Sepharose (Sigma) for 9E10. Antibody was then added (12CA5, 3.8 μg/ml or 9E10 2.7 μg/ml) followed after 2–4 h by 12.5 μl of protein A- or protein G-Sepharose; complexes were precipitated by gentle agitation at 4 °C overnight. The beads were then washed in TDI buffer and either eluted with SDS sample buffer at 65 °C for 5 min or used directly for mannosyltransferase assays.

For preparative scale isolation of protein A fusions, spheroplasting and lysis was carried out as above using 1000 A600 units of cells with the volumes of buffers scaled up accordingly. After lysis and subsequent pelleting of cell debris at 14,000 × g, the supernatant was removed, and 10 μl of protein A-Sepharose (Sigma) was added and incubated for 2 h. The beads were then resuspended in 50 μl of TDI buffer containing 10 units of TEV protease (Life Technologies, Inc.) and rocked overnight at 4 °C. Cleaved product was separated from the beads by pelleting the beads in a microfuge and removing the supernatant. This washing was repeated several times until no beads were observed. For Endo H treatment, the samples were diluted to 1 × SDS sample buffer and heated at 65 °C for 5 min. The denatured samples were then diluted 5-fold and made 50 mM phosphate-citrate (pH 5.9), 0.1% Nonidet P-40. 500 units of Endo H (New England Biolabs) was added, and the mixture was incubated at 37 °C for 1 h. Protein was then precipitated by methanol/chloroform extraction, resuspended in SDS buffer by sonication, and separated by SDS-polyacrylamide gel electrophoresis. After Coomassie staining, gel slices were excised and digested with trypsin, and the tryptic fragment masses were determined by matrix-assisted laser desorption ionization mass spectrometry using a Perceptive Voyager mass spectrometer (39).

Mannosyltransferase Assays—Mannosyltransferase assays on beads were essentially a modified version of that used by Nakajima and Ballou, as described previously (24, 40). Briefly, protein A fusions precipitated on IgG-Sepharose beads were washed once with TDI buffer (4 °C, 15 min) and once briefly with buffer II (50 mM HEPES, pH 7.2, 0.4% digitonin, 10 mM MclCl). Then, 50 μl of buffer II was added to the beads, supplemented by 0.6 mM GDP-α-mannose, 10 mM α-1,6-mannobioside (Dextra Labs), 62 nM GDP-α-U-1,1’-galactoside (Amersham Pharmacia Biotech), and the mixture was gently agitated for 2 h at 30 °C. For reactions lacking Mn2+, MnCl2 was also omitted from the buffer II wash. Reactions were terminated by applying the supernatant of the mixture to a 0.5 × 5-cm Dowex I-X8 column, eluting the neutral reaction products with 800 μl of water, and their radioactivity was quantified by liquid scintillation counting.

RESULTS

Identification of Candidate Genes for MNN2 and MNN5—Neither the MNN2 gene nor the MNN5 gene has been correlated to an ORF in the sequence of the complete yeast genome. However, the mnn2 locus was mapped to chromosome II, between the centromere and gal1 (41). The nucleotide sequence of this section of chromosome II reveals 19 ORFs, and we examined them for homology to known glycosyltransferases. Only ORF YBR015c has such a homology, a distant relationship to Mnn1p, an α-1,3-mannosyltransferase of the yeast Golgi apparatus (Fig. 1B). Like Mnn1p, the protein encoded by YBR015c has a single amino-terminal transmembrane domain and a predicted type II orientation, as seen for all known Golgi glycosyltransferases in both mammals and yeast. Indeed, when this ORF was sequenced as part of the genome project, it was also given the description TTP1 for type II protein (42). Mnn1p has three close homologues in the yeast genome of unknown function, and searching the databases with the sequence of YBR015c revealed homology to these, but also to a second ORF, YJL186w, that was much closer to YBR015c than the Mnn1p family. In addition, there is an ORF in Pichia pastoris that is closer to YBR015c and YJL186w than to MNN1 (Fig. 1B).

Finally, it has been observed recently that the activity of Mnn1p is stimulated by prokaryotic cofactors containing GM (26). This motif is found to be conserved in many families of glycosyltransferases, and it is also conserved in YBR015c and YJL186w (Fig. 1B). These observations suggested that YBR015c was a good candidate to be MNN2, and as mnn2 and mnn5 both affect the addition of an α-1,2-linked mannose, it seemed possible that the closely related ORF YJL186w might be MNN5.

Deletion of YBR015c and YJL186w Results in Defects in Glycosylation—To examine the possibility that the ORFs YBR015c and YJL186w correspond to MNN2 and MNN5, we
made disruptants of the genes in a haploid background and examined the resulting phenotypes. Both deletion strains were viable with no obvious growth defect. One characteristic of the mnn2, but not the mnn5, mutation is that the loss of branches on the α-1,6-linked backbone results in greatly increased binding by anti-α-1,6-mannose antisera (31). Fig. 2 shows that when total proteins from the stains are separated on a protein gel, blotted, and probed with an anti-α-1,6 antisera, greatly increased antibody binding is seen with the DYBR015c strain. We also examined the mobility of the protein invertase in the deletion strains. The N-linked glycans of invertase are modified with hypermannose structures, and the invertase in the DYBR015c strain showed a slightly increased mobility on an SDS gel, consistent with a defect in the N-linked mannan (Fig. 2A). Both mnn2 and mnn5 strains are known to show loss of binding of the cationic dye alcian blue (31, 43). This is due to an absence of phosphate in the mannan structure, apparently which is not shown). The conserved DXD motif found in many families of glycosyltransferases is marked with black circles (26).

Fig. 2. Deletion of ORFs YBR015c and YJL186w results in phenotypes similar to mnn2 and mnn5 and failure to complement these mutations. A, immunoblots of total proteins from either 6210 (WT) or from derivatives in which either YBR015c or YJL186w was deleted, were probed with antibodies to α-1,6-mannose or to the Myc-epitope to detect a Myc-tagged version of invertase. B, anti-α-1,6-mannose immunoblots of total proteins from yeast strains mnn2, its parent X2180, or diploids from matings between mnn2 and the 6210-derived haploid strains in A. C, alcian blue-stained whole cells from the strains in A and B and also the analogous mnn5 haploids and diploids. Equal numbers of stained cells were filtered onto nitrocellulose, washed, and imaged.
caused by the branches being too short for recognition by the phosphomannose-transferase. Fig. 2C shows that the staining of cells by alcian blue is greatly reduced by deletion of either YBR015c or YJL186w. Thus, deletion of YBR015c results in an exposed α-1,6 backbone and a reduction in cell wall phosphate, whereas deletion of YJL186w causes only the latter defect, the same phenotypes seen with mnn2 and mnn5, respectively.

YBR015c and YJL186w Are the Genes MNN2 and MNN5, Respectively—To demonstrate that YBR015c and YJL186w correspond to MNN2 and MNN5, complementation tests were carried out. The deletion strains were mated with mnn2 and mnn5 strains, and the resulting diploids were examined for glycosylation defects. Fig. 2 shows that mating the mutant strains to wild type results in restoration of normal glycosylation as expected from the recessive nature of the mnn2 and mnn5 mutations. In contrast, diploids from the mating of mnn2 and YJL186w carry out. The deletion strains were mated with mnn2 and mnn5 strains, and the resulting diploids were examined for glycosylation defects. Fig. 2 shows that mating the mutant strains to wild type results in restoration of normal glycosylation as expected from the recessive nature of the mnn2 and mnn5 mutations. In contrast, diploids from the mating of mnn2 and mnn5 strains, and the resulting diploids were examined for glycosylation defects. Fig. 2 shows that mating the mutant strains to wild type results in restoration of normal glycosylation as expected from the recessive nature of the mnn2 and mnn5 mutations. In contrast, diploids from the mating of mnn2 and mnn5 strains, and the resulting diploids were examined for glycosylation defects. Fig. 2 shows that mating the mutant strains to wild type results in restoration of normal glycosylation as expected from the recessive nature of the mnn2 and mnn5 mutations. In contrast, diploids from the mating of mnn2 and mnn5 strains, and the resulting diploids were examined for glycosylation defects. Fig. 2 shows that mating the mutant strains to wild type results in restoration of normal glycosylation as expected from the recessive nature of the mnn2 and mnn5 mutations. In contrast, diploids from the mating of mnn2 and mnn5 strains, and the resulting diploids were examined for glycosylation defects. Fig. 2 shows that mating the mutant strains to wild type results in restoration of normal glycosylation as expected from the recessive nature of the mnn2 and mnn5 mutations. In contrast, diploids from the mating of mnn2 and mnn5 strains, and the resulting diploids were examined for glycosylation defects. Fig. 2 shows that mating the mutant strains to wild type results in restoration of normal glycosylation as expected from the recessive nature of the mnn2 and mnn5 mutations. In contrast, diploids from the mating of mnn2 and mnn5 strains, and the resulting diploids were examined for glycosylation defects. Fig. 2 shows that mating the mutant strains to wild type results in restoration of normal glycosylation as expected from the recessive nature of the mnn2 and mnn5 mutations. In contrast, diploids from the mating of mnn2 and mnn5 strains, and the resulting diploids were examined for glycosylation defects.

The Gene Products of MNN2 and MNN5 Are Glycoproteins of the Golgi Apparatus—To further characterize the proteins encoded by MNN2 and MNN5, triple HA epitope tags were directly inserted at the COOH terminus of the genes in the genome by homologous recombination. Immunoblots of total cellular proteins revealed that the two proteins were expressed at comparable levels (Fig. 3A). Mnn2p ran as two closely migrating bands, the upper of which disappeared upon treatment with Endo H, indicating partial use of one of three potential sites for N-linked glycosylation. Mnn5p ran as a single band that shifted 6kJD upon Endo H treatment, suggesting use of at least three of the four potential glycosylation sites. After Endo H treatment, a minor slower migrating band was also visible, suggesting a small degree of modification by O-linked glycosylation. The apparent molecular weights of deglycosylated Mnn2p-HA and Mnn5p-HA (72 and 69 kDa) correspond closely to their predicted sizes (71.8 and 71.3 kDa, respectively).

Examination of the intracellular localization of Mnn2p and Mnn5p by immunofluorescence showed that both proteins were distributed in a punctate pattern characteristic of yeast Golgi (Fig. 3B). Double label immunofluorescence showed that for both proteins, these spots showed substantial colocalization with the cis Golgi protein Anp1p, but not with the medial Golgi proteins Mnn1p and Mnt1p (Fig. 3B and data not shown). The distribution of Mnn2p and Mnn5p were compared by tagging them with different epitope tags, and a high degree of colocalization was observed. The distribution of Anp1p and of Mnn2p or Mnn5p was never absolutely identical, with occasional spots being positive for only one or the other marker. A similar situation has been observed for other yeast Golgi proteins in both the cis and medial compartments, and this presumably reflects the rapid recycling and movement of resident proteins within the Golgi stack (24, 44–46).

Investigation of Possible Physical Interactions between Mnn2p and Mnn5p and Other Golgi Proteins—The Golgi proteins involved in synthesizing the α-1,6-linked mannann backbone are found in two multi-protein complexes, which share Mnn9p as a common component (24). Since Mnn2p and Mnn5p are apparently required for the next steps in mannan synthesis, we were interested to determine if these proteins are associated with either of these Mnn9p-containing complexes or, indeed, with any other Golgi proteins. Thus, the epoite-tagged Mnn2p and Mnn5p were immunoprecipitated from cells lysed with the mild detergent digitonin, and the immunoprecipitates were blotted for the presence of known Golgi enzymes. Fig. 4A shows that precipitation of Mnn2p or of Mnn5p did not result in the coprecipitation of either Van1p or Anp1p, components of the two complexes that synthesize the α-1,6-linked backbone. Likewise, neither Mnn2p nor Mnn5p showed association with either Mnn1p, which adds the α-1,3-linked mannoe to the α-1,2-linked mannoe that is missing in the mnn5 mutant, or with Mnt1p, an α-1,2-mannosyltransferase that modifies O-
linked glycans. Finally, examination of a strain expressing epitope-tagged forms of both Mnn2p and Mnn5p showed that when one is precipitated, the other does not coprecipitate (Fig. 4B). Thus, it seems that neither Mnn2p nor Mnn5p associates with other Golgi enzymes involved in mannan synthesis.

To investigate whether these proteins have any binding partners, a different system for precipitation was used that gives low background even with mild detergent conditions. To achieve this, a protein A tag was inserted at the COOH terminus of both MNN2 and MNN5 (Fig. 5A). The linker between the ORF and the protein A contains a cleavage site for the sequence-specific protease from TEV (34). Thus, the protein A fusions can be precipitated from digitonin-solubilized cells on IgG-Sepharose, washed, and then eluted with TEV protease under non-denaturing conditions. This elution method releases the tagged protein and any binding partners while leaving behind the proteins stuck nonspecifically to the beads, thereby minimizing the background. Fig. 5B shows the results of such a precipitation performed with protein A-tagged Mnn2p and Mnn5p. When the eluted proteins were separated by SDS-polyacrylamide gel electrophoresis and stained with Coomassie Blue, a single predominant band was observed in both cases, suggesting that neither protein is stoichiometrically associated with a second component. For both proteins, an additional faint band could be seen that was specific to the precipitated protein (Fig. 5B). To identify these bands and to confirm the identity of the major band, they were excised from the gel and digested in the gel slice with trypsin, and the tryptic peptides were analyzed by mass spectrometry. The masses of the tryptic fragments confirmed that the major bands were indeed Mnn2p and Mnn5p. In addition, the minor band migrating ahead of Mnn2p produced tryptic peptides characteristic of Mnn2p, suggesting that it is a proteolytic product from Mnn2p that has probably arisen during precipitation, as it is not seen in the blot of total protein from yeast expressing tagged Mnn2p (Fig. 4A). The minor band migrating behind Mnn5p also produced fragments characteristic of Mnn5p, and this presumably corresponds to the minor band seen in this position in the total protein blot in Fig. 4A.

**Fig. 4.** Mnn2p or Mnn5p do not interact with known Golgi enzymes involved in mannan synthesis. A, immunoblots of proteins isolated by anti-HA immunoprecipitation from strains in which the triple HA epitope was inserted at the COOH terminus of the ORFs for the indicated proteins. The blots were probed with antisera against Van1p or Anp1p as shown. B, immunoblots of proteins isolated by anti-epitope tag immunoprecipitation from strains coexpressing two proteins tagged with the Myc (M) or the HA (H) epitopes. The tagged proteins were Mnn2p (2), Mnn5p (5), Mnn1p (1), and Mnt1p (T), the precipitations were performed with mouse monoclonals, and then the blots were probed with rabbit anti-tag sera as shown.

**Fig. 5.** Mnn2p and Mnn5p do not form a stoichiometric association with any other protein. A, structure of the protein A fusion chimeras. The open reading frame is extended by a linker containing the cleavage site for TEV protease and then two copies of a synthetic "Z" domain from protein A. B, Coomassie-stained gel of the proteins eluted by TEV protease from IgG-Sepharose beads incubated in digitonin lysates of the indicated strains ("control" is 6210 containing no fusion protein). The proteins were digested with Endo H following elution, and each lane represents the product of 1000 A600 units of cells. The 30-kDa band in all lanes is the protease, and the 90-kDa band from wild-type 6210 is a sporadically observed background band.
Thus, both the Mnn2p and Mnn5p proteins have mannosyltransferase activity in vitro. Mannosyltransferase activity from GDP-mannose to α-1,6-mannobiose was assayed in vitro on IgG-Sepharose beads precipitated from strains expressing the indicated protein A fusions. The assay was performed as described under "Experimental Procedures" (complete) and also with either the acceptor or the manganese, omitted as indicated. The results are means of triplicates with error bars showing the standard deviation.

Thus, it seems that neither Mnn2p nor Mnn5p associates stoichiometrically with any other proteins in the same mild detergent conditions that have allowed the isolation of complexes of membrane proteins from both ER and Golgi. Such a protein A fusion has been used successfully to isolate the large complex of Golgi proteins containing Anp1p.2 This suggests that Mnn2p and Mnn5p perform their functions separately and not as part of a larger complex.

Mnn2p and Mnn5p Have Mannosyltransferase Activity—The homology of the sequences of Mnn2p and Mnn5p with the known mannosyltransferase Mnn1p, and the loss of a specific linkage in the mnn2 and mnn5 mutants, strongly suggests that the proteins are themselves mannosyltransferases. To investigate this, the protein A chimeras described above were precipitated, and mannosyltransferase activity on the beads was assayed using GDP-mannose and α-1,6-mannobiose as an acceptor. Fig. 6 shows that Mnn2p-protein A has mannosyltransferase activity in this in vitro assay, and this was inhibited by removing the divalent cation manganese. The Mnn5p-protein A fusion showed a smaller amount of transferase activity in the same assay, which presumably reflects the fact that its normal substrate would be a branched version of the α-1,6 backbone. Thus, both the Mnn2p and Mnn5p proteins have mannosyltransferase activity in vitro.

**DISCUSSION**

In this paper, we have identified the yeast genes corresponding to the mannann synthesis mutants mnn2 and mnn5. The proteins encoded by these genes are both predicted to be type II membrane proteins, and both are primarily localized to the cis Golgi. Our immunoprecipitation experiments demonstrate that Mnn2p and Mnn5p exist as discrete proteins rather than associating with any of the other enzymes involved in synthesizing the mannann structure. Previous characterization of mnn2 and mnn5 strains has shown that in each case a specific linkage in the N-linked mannann structure is missing, strongly suggesting that the MNN2 and MNN5 gene products are mannosyltransferases (30, 31). Consistent with this, the gene products show homology to the Golgi α-1,3-mannosyltransferase Mnn1p. This homology includes an aspartate-containing motif that we have recently shown to be essential for the catalytic activity of Mnn1p and that is conserved in a diverse range of both prokaryotic and eukaryotic glycosyltransferases (26). Moreover, mannosyltransferase activity was detectable in vitro with precipitated fusion proteins of Mnn2p and Mnn5p. Thus, the simplest hypothesis for the function of Mnn2p and Mnn5p is that they are the two α-1,2-mannosyltransferases that act sequentially to form, and then extend, the branches on the mannann backbone.

*S. cerevisiae* contains a second family of well characterized α-1,2-mannosyltransferases, the closely related Golgi proteins Mnt1p, Ktr1p, and Ktr3p that are required for attaching the second and third mannoses to O-linked glycans (47). Although the residues they add are both α-1,2-linked mannoses, these enzymes show no sequence homology to Mnn2p or Mnn5p. It has been reported that when MNT1, KTR1, and KTR3 are all deleted, not only is extension beyond the first O-linked mannann absent, but in addition a small increase in the mobility of invertase is observed, suggesting that N-linked structures are also affected (48). It is possible that extension of the mannann branches can also be performed to some extent by the Mnt1p family, either contributing to the addition of the second mannose or possibly adding a third α-1,2-linked mannose, as has been reported for branches in other yeasts (9). Alternatively, the effect could be indirect if some of the mannann-synthesizing enzymes require full-length O-linked chains to be fully effective in the Golgi. α-1,2-Linked mannann is also found on GPI anchors, but this is unaffected in mnn2 and mnn5 mutants (49).

The identification of the Mnn2p and Mnn5p mannosyltransferases means that it is now possible to assign Golgi enzymes to all of the catalytic steps in the formation of the *S. cerevisiae* mannann structure (Fig. 7). The initial extension and subsequent elongation of the backbone are catalyzed by two multi-protein complexes, but these complexes do not contain the enzyme acting before this step, Och1p, nor, as shown here, the subsequent transferases Mnn2p and Mnn5p. Likewise, Mnn2p and Mnn5p do not seem to associate with each other or with the Mnn1p enzyme that catalyzes the next step in elaboration of the mannann branch, acting on the two α-1,2-linked mannoses whose addition requires first MNN2 and then MNN5. Thus, it seems that the enzymes involved in branch formation act separately and not as a complex. It is perhaps noteworthy that the three proteins that are required to make the basic branch, Mnn2p, Mnn5p, and Mnn1p, share sequence homology. This suggests that these enzymes may have evolved from a common ancestor that made a simple branch structure, and subsequent evolutionary selection for branch variation has acted on duplicated genes to produce a diversity of transferases. A requirement for continuous variation of the branch-forming enzymes during evolution may have prevented them forming a larger complex of the type seen for the more conserved α-1,6-linked backbone.

There are several reasons why these branch-forming enzymes in *S. cerevisiae* may be of wider interest. First, the mannann branches are the structures that are recognized during immune responses against pathogenic yeast. This, combined with the possible involvement in adhesion during infection, means that these enzymes are potential targets for antifungal drugs (8). Second, flocculation of *S. cerevisiae* is mediated through lectin-like recognition of the mannann branches and so is dependent on the activity of MNN2 (7). Thus, regulation of the expression of MNN2 might provide a means of controlling flocculation, a critical process in fermentation (50). Third, the fact that two related proteins, Mnn2p and Mnn1p, are located in different cisternae of the Golgi may provide a system to investigate the general question of how the enzymes of the Golgi are restricted to particular subcompartments of this structure (51). Finally, there is evidence that the mannann-synthesizing enzymes interact with signal transduction pathways. Several enzymes in Fig. 7 show genetic interactions with the signaling pathways that are involved in mon-

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2 J. C. Rayner and S. Munro, unpublished data.
itoring cell wall integrity or in regulating the cell cycle. Thus, 
HOC1 is a multicyclic suppressor of mutations in protein kinase 
C (PKC), and loss of any of ANP1, MNN9, and OCH1 produces 
synthetic lethality with overexpression of a cyclin that by-
passes a cell-cycle checkpoint at budding (52, 53). Similar ge-
netic interactions have been described for the enzymes that are 
involved in the synthesis of the glucan and chitin components 
of the cell wall, such as Krc6p and Chs3p (54, 55). It seems that 
when one component of the cell wall is defective, signal trans-
duction pathways are required to induce compensatory 
changes in the synthesis or targeting of other cell wall com-
ponents (56). Thus, these interactions of the mannan-synthesiz-
ing enzymes presumably reflect the importance of the mannan 
structure for cell wall integrity. Interestingly, there is evidence 
that loss of activity of MNN2 is also sensed by one of the cell 
wall-sensitive signaling pathways. A genetic screen was re-
cently reported identifying mutations with a Ca^{2+}-resistant 
and vanadate-sensitive (crv) phenotype (57). Four such crv 
mutations were obtained, one of which, crv4, was identified as 
yielding YBR015c, which we have now shown corresponds to 
MNN2. It was previously known that loss of the Ca^{2+}-regu-
lated protein phosphatase calcineurin in yeast results in a crv 
phenotype, and crv1 is allelic to cnp1, the regulatory subunit of 
calcineurin. Interestingly, CRV2 and CRV3 were shown to be 
BCK1 and SLT2, components of the mitogen-activated protein 
kinase pathway that is regulated by PKC and that senses cell 
wall integrity (58). Moreover, a double mutation of crv4 (i.e. 
{mnn2}) and slt2 showed a more severe osmotic sensitivity than 
either mutation alone, suggesting that unbranched mannan 
expressed in an mnn2 strain results in an altered cell wall and 
that signaling via the PKC pathway is required to compensate 
for this defect. Evidence is beginning to emerge for the down-
stream targets of these signal transduction pathways that al-
low compensation for cell wall defects. Thus, it seems that 
the transcription of MNN1 and KRE6 is regulated in part by PKC 
(55), but it is also possible that there is post-transcriptional 
control of the activity or stability of the Golgi enzymes. Now 
that most of the enzymes required for mannan synthesis in the 
yeast Golgi have been identified, it should be possible to un-
derstand more of how they are organized within the Golgi and 
to determine how their activity is regulated by growth state, 
cell cycle, and cell wall integrity.

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