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Regulation of the protein glycosylation pathway in yeast: Structural control of N-linked oligosaccharide elongation

(*Saccharomyces cerevisiae* mannoproteins/mannosyltransferase/invertase/GDP-mannose/^{35}S incorporation

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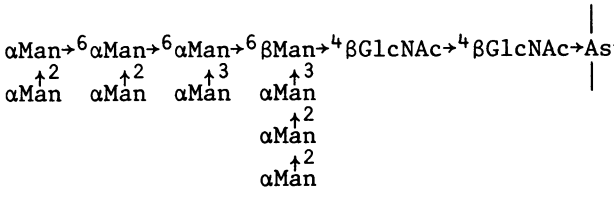
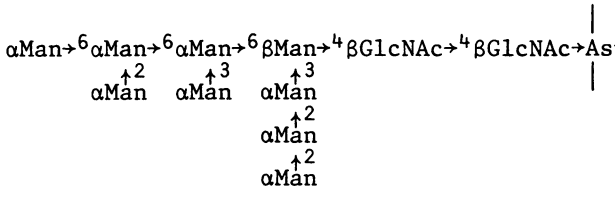
ABSTRACT **The yeast *Saccharomyces cerevisiae* X2180 strain with the *mnn1 mnn2 mnn9* mutations, all of which affect mannoprotein glycosylation, synthesizes N-linked oligosaccharides having the following structure:**



whereas the *mnn1 mnn2* mutant extends the α1→6-linked backbone of some of the core oligosaccharides by adding 20–30 mannose units. Membrane fractions from the *mnn1 mnn2* and *mnn1 mnn2 mnn9* mutants are equally effective in catalyzing transfer from GDP-[^{3}H]mannose to add mannose in both α1→2 and α1→6 linkages to an oligosaccharide having the following structure:



but neither membrane preparation can utilize the homologous *mnn1 mnn2 mnn9* oligosaccharide as an acceptor. Thus, addition of the α1→2-linked mannose side chain to the terminal α1→6-linked mannose in oligosaccharides of the *mnn9* mutant inhibits the elongation reaction and may serve as an important structural control of mannoprotein glycosylation. The *mnn9* mutation also increases the transit time for invertase secretion, meaning that this mutation could affect the processing machinery in the Golgi apparatus.

Several mutants have been obtained in this laboratory that affect the glycosylation of proteins in *Saccharomyces cerevisiae* (1–5). One of these, designated *mnn9* (4, 6), allows addition of a single α1→6-linked mannose to the endoplasmic reticulum-processed core oligosaccharide $Man_8GlcNAc_2$-, but it prevents further elongation of the backbone to form the typical wild-type polymannose outer chain. In the *mnn1 mnn9* mutant, all N-linked oligosaccharides accumulate as $Man_{10}GlcNAc_2$- units in which a second mannose is added in α1→2 linkage to the terminal α1→6-linked mannose (6). Here we show that this modified core oligosaccharide is inactive as an acceptor with GDP-[^{3}H]mannose as donor and a membrane-bound mannosyltransferase preparation (7) from the *mnn1* or *mnn1 mnn9* strains, whereas the homologous oligosaccharide with an unsubstituted terminal α1→6-linked mannose (8, 9) is a good acceptor of mannose to form both α1→2 and α1→6 linkages. The results suggest that the 2-substitution of the terminal α1→6-linked mannose unit serves as a stop signal that controls outer chain addition.

EXPERIMENTAL PROCEDURES

Materials. *S. cerevisiae* X2180 and the glycosylation mutants *mnn1 mnn2*, *mnn1 mnn2 mnn9*, and *mnn1 mnn9* were kindly provided by Lun Ballou. Yeast cultures were grown in liquid YEPD (1% yeast extract/2% Bacto-peptone/2% D-glucose) at 30°C on a rotary shaker. Cultures were maintained on 2% agar plates of YEPD. GDP-[1-^{3}H]mannose (10 Ci/mmol; 1 Ci = 37 GBq) and EN^{3}HANCE were purchased from New England Nuclear, and $H_2{}^{35}SO_4$ (16 Ci/mmol) was from Amersham. IgGsorb was obtained from The Enzyme Center (Boston); Dowex AG 1-X8, Dowex 501-X8, and Bio-Gel P-2, P-4, and P-6 (all 400 mesh) were from Bio-Rad; and GDP-mannose and other chemicals were from Sigma.

Zymolase 5000 was supplied by Kirin Brewery (Japan). α1→2-Mannosidase was purified from *Aspergillus saitoi* (10) and α1→6-endomannanase was from *Bacillus circulans* (11). Endoglucosaminidase H (12) was a gift from Lun Ballou. α1→6-Mannooligosaccharides were prepared from mannan isolated from the *mnn2* mutant by partial digestion with α1→6-endomannanase (11) or by partial acetolysis (13). Core oligosaccharide acceptors were isolated from *mnn1 mnn2* (9) and *mnn1 mnn2 mnn9* (6) mutants.

Isolation and Solubilization of Mannosyltransferase. *S. cerevisiae* cells were grown to midlogarithmic phase in 2-liter Fernbach flasks and were washed twice with cold 1% KCl by centrifugation. Cell paste was resuspended in 0.1 M Tris·HCl buffer (pH 7.2) containing 10 mM $MnCl_2$, 10 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, and 5% glycerol (homogenizing buffer), and glass beads were added to this suspension (1:2:2, wt/vol/wt). Cells were broken in a Braun homogenizer at 2–4°C with three 1-min pulses. Unbroken cells and beads were removed by centrifugation at 5000 rpm for 10 min, and the mixed membrane fraction was isolated from the supernatant liquid by centrifugation at 100,000 × *g* for 90 min. The isolated membrane pellet was resuspended in the homogenizing buffer to a protein concentration of 10 mg/ml, Triton X-100 was added to a final concentration of 2% (wt/vol), and the mixture was stirred at 4°C for 18 hr. Solubilized membrane proteins were recovered by a second centrifugation at 100,000 × *g* for 90 min. Such preparations retained transferase activity for at least 1 month when stored at 4°C, but they were normally discarded after 1 week.

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Mannosyltransferase Assay. The standard assay was carried out essentially as described by Nakajima and Ballou (7). In a total volume of 50 μl, the incubation mixture contained 50 mM Tris·HCl (pH 7.2), 10 mM $MnCl_2$, 2 mM exogenous acceptor, 0.6 mM GDP-[^{3}H]mannose (50,000–150,000 cpm), and solubilized enzyme preparation (20–50 μg of protein). The reaction mixture was incubated at room temperature (20–23°C) for 30–60 min and the reaction was stopped by pouring the mixture over a Dowex AG-1 (acetate) column (0.5 ml in a Pasteur pipet), after which the neutral product was eluted with water. Radioactivity was counted with Scint A. Controls were done without added acceptor.

For product analysis, reactions were scaled up 5- to 20-fold and the products were purified on a Bio-Gel P-4 column (1 × 180 cm) that was calibrated with oligosaccharide standards. Pooled fractions of each product were acetolyzed (14), and the recovered acetylated material was dried under N_2, deacetylated, and desalted on a Dowex 501-X8 mixed-bed ion-exchange column before thin-layer chromatography. Precoated cellulose thin-layer plates, used to separate the products of acetolysis, were developed three times in pyridine/ethyl acetate/water, 3:5:2 (vol/vol), and the relative mobilities of standards were detected by staining the plates with alkaline silver nitrate.

Active receptor was prepared from the *mnn1 mnn2 mnn9* core oligosaccharide by mannosidase digestion. In a total volume of 50 μl, the incubation mixture contained 40 mM sodium acetate (pH 4.5), 0.5 mM zinc acetate, 1 mg of oligosaccharide, and 10 μl of α1→2-mannosidase (0.1 unit/ml). The mixture was incubated at 30°C under toluene for 10 hr, after which it was fractionated on a Bio-Gel P-4 column to recover the modified oligosaccharide.

To assay the acceptor activity of such modified oligosaccharides, 20 nmol of the oligosaccharide was mixed with 120 nmol of GDP-[^{3}H]mannose (150,000 cpm per assay), and the solution was evaporated to dryness under a stream of N_2. Solubilized enzyme, 40 μg of protein in 20 μl of homogenization buffer, was added and the mixture was incubated at room temperature (20–23°C) for 1 hr; the product was then recovered and analyzed as above.

^{35}S Labeling and Immunoprecipitation of Invertase. Cells were grown to midlogarithmic phase in minimal medium containing 5% glucose, 0.1 mM ammonium sulfate, and vitamins. Ten OD_{600} units of cells from this culture was placed in a medium for derepression of invertase synthesis (0.1% glucose in minimal medium without sulfate). After 30 min, carrier-free $H_2{}^{35}SO_4$ (25 μCi) was added and the cells were incubated for 5 min. Cultures were then adjusted to 0.5% glucose, 1 mM sulfate, and 100 μg of cycloheximide per ml to initiate chase of the ^{35}S label. Samples (2 OD_{600} units per time point) were collected at intervals into chilled tubes containing 10 μl of 1 M sodium azide. The cells were washed once with 10 mM chilled azide by centrifugation and resuspended in 0.2 ml of 1.8 M sorbitol in 50 mM potassium phosphate buffer (pH 7.5) containing 10 mM azide, 80 mM 2-mercaptoethanol, and 50 μl of Zymolyase 5000 (10 mg/ml).

Cells were incubated at 37°C for 45 min to obtain spheroplasts, which were separated by centrifugation at 3000 × *g* for 10 min. Fifty microliters of 10% sodium dodecyl sulfate was added to the pellet and supernatant fractions, and each was boiled for 3 min and then adjusted to 1 ml with phosphate-buffered saline containing 2% Triton X-100. These two fractions were then treated with 50 μl of IgGsorb, prepared as described in the supplier's manual. After incubation at 0°C for 30 min, the IgGsorb was removed by centrifugation on a Microfuge for 20 min at 12,000 × *g*. The supernatant fractions thus obtained were transferred to new Microfuge tubes, 4 μl of anti-invertase serum was added, and the tubes were left on ice at 4°C overnight. IgGsorb (50 μl) was added, and the immunocomplexes formed after a 30-min incubation at 0°C were sedimented at 12,000 × *g* for 10 min. Precipitated complexes were washed twice with 0.5 ml of 2 M urea in 0.1

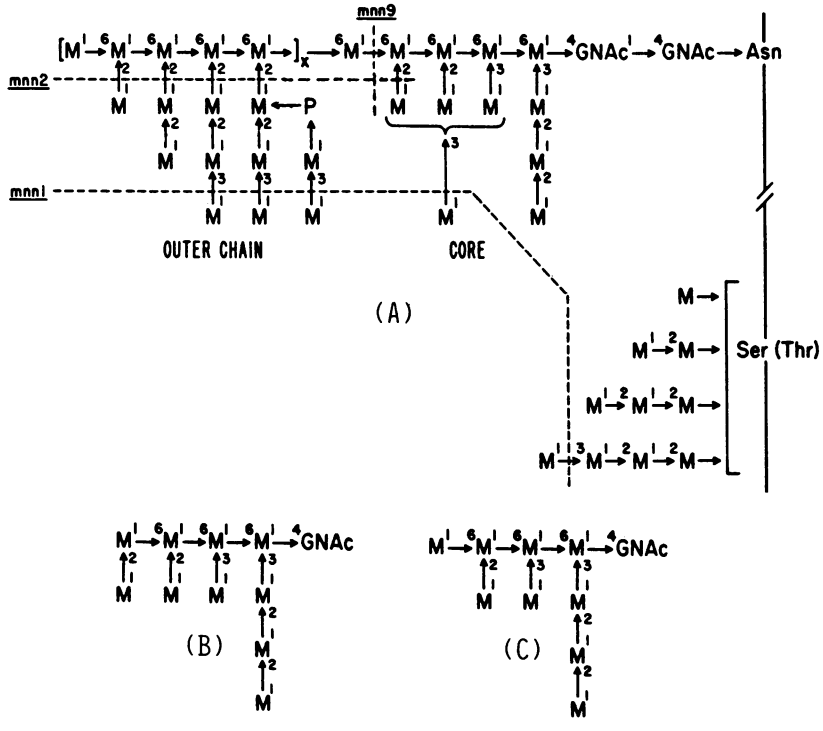

FIG. 1. Carbohydrate structure of *S. cerevisiae* mannoproteins. GNAc, GlcNAc; M, Man. (*A*) Structure of the N- and O-linked oligosaccharides of the wild-type strain with dashed lines to indicate those parts of the molecule that are eliminated by three of the *mnn* mutations. The crossing of the lines for *mnn2* and *mnn9* indicates that, in the absence of *mnn9*, the *mnn2* defect extends into the core, whereas in the presence of *mnn9* it does not. (*B*) Structure of the oligosaccharide released from *mnn1 mnn2 mnn9* mannoprotein by digestion with endoglucosaminidase H. (*C*) Structure of the major oligosaccharide obtained by endo-α1→6-mannanase digestion of the oligosaccharide released from *mnn1 mnn2* mannoprotein by endoglucosaminidase H. A minor component has one additional α1→6-linked mannose on the backbone.

M Tris·HCl (pH 7.5) containing 0.2 M NaCl and 1% Triton X-100 and washed twice with 0.5 ml of 1% 2-mercaptoethanol. Final pellets were resuspended in 30 μl of denaturing buffer (50 mM Tris·HCl, pH 6.8/10% glycerol/1% sodium dodecyl sulfate/2% 2-mercaptoethanol), boiled for 3 min, and electrophoresed on a 7.5% polyacrylamide slab gel according to Laemmli (15). Gels were fixed in 25% isopropyl alcohol containing 10% acetic acid, treated with EN^{3}HANCE, washed, dried, and exposed to Kodak XAR film at −70°C. Autoradiograms were analyzed by scanning densitometry.

RESULTS AND DISCUSSION

***S. cerevisiae mnn1 mnn2* and *mnn1 mnn2 mnn9* Mannoproteins Have Different Core Structures.** A generalized structure for the carbohydrate chains of *S. cerevisiae* X2180 mannoproteins is shown in Fig. 1*A*; the parts of the molecule that are eliminated in the *mnn1*, *mnn2*, and *mnn9* mutants are indicated by the dashed lines. The *mnn1* mutation prevents addition of terminal α1→3-linked mannose, and the *mnn9* mutation prevents elongation of the outer chain, so that the *mnn1 mnn9* double mutant has N-linked oligosaccharides with the composition $Man_{10}GlcNAc_2$- (Fig. 1*B*). Since the *mnn2* mutation affects addition of side chains only to the outer chain, the N-linked oligosaccharides in a *mnn1 mnn2* double mutant have a long unsubstituted α1→6-linked polymannose unit attached to the core (8). The *mnn1 mnn2* core, however, is not identical to that found in the *mnn1 mnn2 mnn9* strain because, in the absence of the *mnn9* defect, the *mnn2* mutation extends into the core and eliminates one of the α1→2-linked mannose units found in the *mnn1 mnn9* core (6, 9). This conclusion derives from a comparison of the endoglucosaminidase H-produced *mnn1 mnn2 mnn9* core oligosaccharide (Fig. 1*B*) with that produced by endo-α1→6-mannanase digestion of the *mnn1 mnn2* oligosaccharide obtained by endoglucosaminidase H digestion of the mannoprotein (Fig. 1*C*). The following experiments suggest that this structural difference is an important factor in regulating elongation of the backbone of N-linked oligosaccharides.

Membranes from *mnn1 mnn2* and *mnn1 mnn2 mnn9* Strains Show Similar Mannosyltransferase Activities. Table 1 compares the relative activities of a number of oligosaccharides as acceptors with GDP-[^{3}H]mannose as donor and a membrane enzyme preparation from the two yeast strains. The radioactive products were analyzed for the proportion of mannose added in α1→2 and α1→6 linkage by partial acetolysis (14), in which reaction the α1→6-linked mannose is converted to ^{3}H-labeled monosaccharide and the α1→2-linked mannose is resistant to acetolysis (7) and is recovered in larger ^{3}H-labeled oligosaccharides. From previous studies, it was found that the α1→2-mannobiose was an excellent acceptor for mannose in α1→2-linkage, whereas the α1→6-mannotriose was a good acceptor for mannose in both α1→2 and α1→6 linkage (7). As shown in Table 1, these results were reproduced, with the first acceptor giving mainly α1→2-linked product, whereas the latter acceptor gave comparable amounts of both types of linkage. The α1→6-mannosyltransferase of *S. cerevisiae* has been shown to prefer inter-

Table 1. Mannosyltransferase activities of core oligosaccharides

Acceptor*	*mnn1 mnn2* membranes			*mnn1 mnn2 mnn9* membranes		
	Mannose incorporated, nmol*	Linkage		Mannose incorporated, nmol*	Linkage	
		α1→2	α1→6		α1→2	α1→6
αM→^{2}αβM	3.04	0.94	0.06	1.83	0.92	0.08
αM→^{6}αM→^{6}αβM	2.55	0.60	0.40	2.61	0.65	0.35
αM→^{6}(αM→^{2})αM→^{6}(αM→^{2}αM→^{3})βM→^{4}αβGNAc	3.35	0.72	0.28	4.47	0.84	0.16
αM→^{6}(αM→^{2})αM→^{6}(αM→^{2}αM→^{3})βM→^{4}αβGNAc	4.30	0.71	0.29	4.23	0.84	0.16
αM→^{6}(αM→^{2}αM→^{2})αM→^{6}(αM→^{2}αM→^{3})βM→^{4}αβGNAc	0.29	ND	ND	0.15	ND	ND
αM→^{6}(αM→^{2})αM→^{6}(αM→^{2}αM→^{3})βM→^{4}αβGNAc	0.09	ND	ND	0.21	ND	ND

ND, not determined. GNAc, GlcNAc; M, Man.
*Acceptor was 2 mM and GDP-mannose was 0.6 mM.

mediate-sized α1→6-mannooligosaccharides, with the acceptor activity decreasing below the trisaccharide and above the hexasaccharide (7).

The *mnn1 mnn2 mnn9* Core Oligosaccharide Is Not an Acceptor when Incubated with GDP-[^{3}H]Mannose and Membranes from *mnn1 mnn2* or *mnn1 mnn2 mnn9* Cells. The results of mannosyltransferase assays with core oligosaccharides from the mutant mannoproteins are listed in Table 1. The acceptor prepared by endomannanase digestion of the *mnn1 mnn2* oligosaccharide, a $Man_9GlcNAc$ with a terminal unsubstituted α1→6-linked mannose, had good activity with the α1→2 and α1→6 transferases. In contrast, the *mnn1 mnn2 mnn9* oligosaccharide, which lacks an unsubstituted α1→6-linked mannose, showed very low activity, if any. Assuming that this oligosaccharide could accept only α1→6-linked mannose, we would expect an incorporation of 1.2 nmol in the standard assay. The small and variable incorporation of [^{3}H]mannose that was observed could be due to endogenous acceptors.

Exo-α1→2-Mannosidase Digestion Converts the *mnn1 mnn2 mnn9* Core Oligosaccharide to an Active Acceptor. The *mnn1 mnn2 mnn9* core oligosaccharide was digested with the exomannosidase, and the products were separated on a Bio-Gel P-4 column (Fig. 2). Two carbohydrate-containing peaks were obtained, one corresponding to mannose and the other corresponding to $Man_6GlcNAc$. This modified core oligosaccharide proved to be an excellent acceptor in the mannosyltransferase assay (Table 2). Similar treatment of the *mnn1 mnn2 mnn9* "large" core oligosaccharide, which retained the terminal α1→2-linked mannose of the lipid-linked precursor (6), gave an acceptor with similar activity.

The Transit Time for Invertase Translocation and Secretion Is Increased in the *mnn9* Mutant. In the wild-type strain of *S. cerevisiae*, the transit half-time from the endoplasmic reticulum to the periplasm is reported to be about 1.5 min, whereas in mutants that are defective in the leader sequence, transit half-times of 10–75 min are observed (16). In the experiments reported here, we found a transit half-time of about 1 min for strain X2180 and the *mnn1 mnn2* mutant, whereas the *mnn1 mnn2 mnn9* mutant gave a value of 5 min (Fig. 3). Although this is not as dramatic an effect on the processing times for yeast glycoproteins as that described by Schauer *et al.* (16), it does indicate that the *mnn9* mutation delays the movement of invertase from the endoplasmic reticulum to the periplasm.

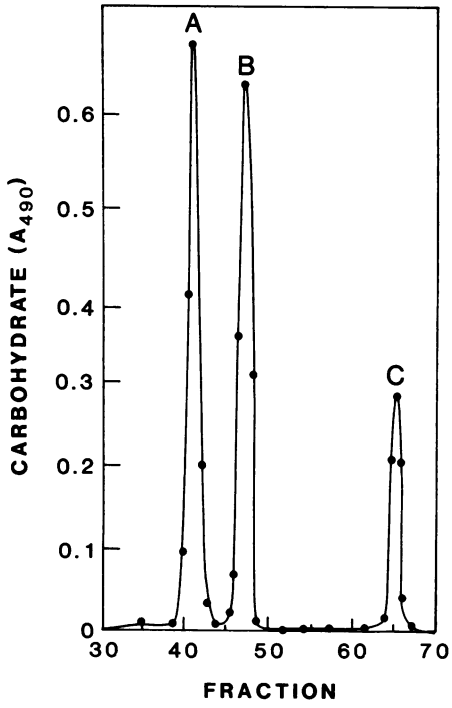

FIG. 2. Digestion of *mnn1 mnn2 mnn9* core oligosaccharide with α1→2-mannosidase. The enzymic digest was fractionated on a Bio-Gel P-4 column, and the elution positions of the starting oligosaccharide $Man_{10}GlcNAc$ (A), the oligosaccharide product (B), and released mannose (C) are shown.

Table 2. Acceptor activity of α1→2-mannosidase-digested oligosaccharides

Acceptor*	Mannose incorporated, nmol*	
	mnn1 mnn2 membranes	*mnn1 mnn2 mnn9* membranes
None	1.50	1.83
mnn1 mnn2 mnn9 small core	1.57	ND
mnn1 mnn2 small core	ND	10.12
α1→2-Mannosidase-digested *mnn1 mnn2 mnn9* small core	10.50	8.90
α1→2-Mannosidase-digested *mnn1 mnn2 mnn9* large core	7.06	5.49

Each assay contained 20 μg of protein. ND, not determined.
*Acceptor, when present, was 1 mM and GDP-mannose was 6 mM.

Conclusions. The *mnn9* mutation in *S. cerevisiae* is of interest because it prevents processing of all N-linked oligosaccharides beyond the stage characteristic of carboxypeptidase Y, a vacuolar glycoprotein. The secreted form of yeast invertase normally has an assortment of N-linked oligosaccharides in which some are unmodified, some are modified as for carboxypeptidase Y, and some are modified by the addition of a long and branched polymannose outer chain (17, 18). The last modification does not occur in the *mnn9* mutant, and our results suggest that this could be the consequence of a defect that incorrectly causes or allows addition of an α1→2-linked mannose that normally serves as a stop signal to prevent further elongation. A striking feature of the *mnn9* mutation is that it is not leaky—that is, it does not allow

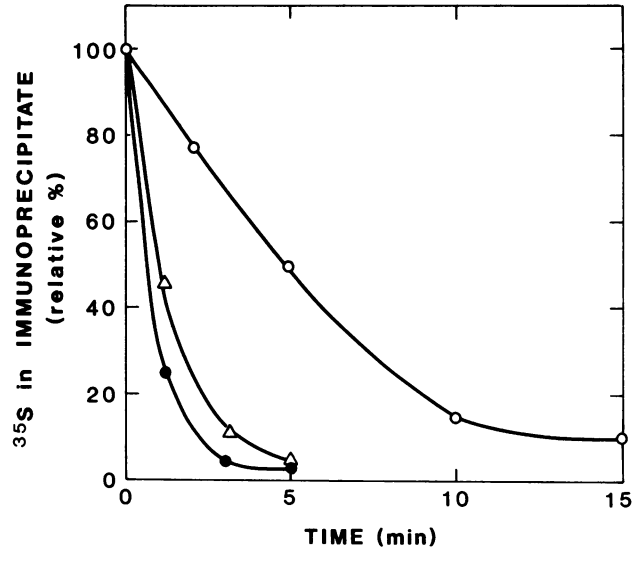

FIG. 3. Kinetics of invertase secretion. After a 5-min incubation with $^{35}SO_4^{2-}$, cells were incubated in the presence of unlabeled sulfate and, at intervals, samples were converted to spheroplasts that were lysed and the invertase was immunoprecipitated. The time-dependent decrease in immunoprecipitable ^{35}S is shown for yeast strains X2180 (wild type) (●), *mnn1 mnn2* (△), and *mnn1 mnn2 mnn9* (○) as % of the initial value. The half-time for strains X2180 and *mnn1 mnn2* was about 1 min, whereas that of the *mnn1 mnn2 mnn9* strain was 5 min.

formation of even a trace of oligosaccharide with outer chain. Because the N-linked chains that accumulate on proteins in the *mnn9* mutant are indistinguishable from those found on carboxypeptidase Y in the wild-type strain (6), we conclude that the defect may act in a processing step at which intracellular and extracellular mannoproteins would normally be treated differently. Although the *mnn9* mutation could have eliminated a specific α1→6-mannosyltransferase, our studies indicate that this is not so, although mislocalization of such an enzyme might lead to the observed phenotype. Conversion of the precursor oligosaccharide $Glc_3Man_9GlcNAc_2$- to $Man_8GlcNAc_2$- occurs in the endoplasmic reticulum (19), and further processing of the latter oligosaccharide apparently occurs in the Golgi apparatus, so the *mnn9* defect could affect a specific function of the latter compartment.

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