

Golgi Localization and *in Vivo* Activity of a Mammalian Glycosyltransferase (Human β 1,4-Galactosyltransferase) in Yeast*

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Gene fusions encoding the membrane anchor region of yeast α 1,2-mannosyltransferase (Mnt1p) fused to human β 1,4-galactosyltransferase (Gal-Tf) were constructed and expressed in the yeast *Saccharomyces cerevisiae*. Fusion proteins containing 82 or only 36 N-terminal residues of Mnt1p were produced and quantitatively N-glycosylated; glycosyl chains were shown to contain α 1,6-, but not α 1,3-mannose determinants, a structure typical for an early Golgi compartment. A final Golgi localization of both fusions was confirmed by sucrose gradient fractionations, in which Gal-Tf activity cofractionated with Golgi Mnt1p activity, as well as by immunocytological localization experiments using a monoclonal anti-Gal-Tf antibody. In an *in vitro* Gal-Tf enzymatic assay the Mnt1/Gal-Tf fusion and soluble human Gal-Tf had comparable K_m values for UDP-Gal (about 45 μ M). To demonstrate *in vivo* activity of the Mnt1/Gal-Tf fusion the encoding plasmids were transformed in an *alg1* mutant, which at the non-permissive temperature transfers short (GlcNAc)₂ glycosyl chains to proteins. Using specific lectins the addition of galactose to several yeast proteins in transformants could be detected. These results demonstrate that Gal-Tf, a mammalian glycosyltransferase, is functional in the molecular environment of the yeast Golgi, indicating conservation between yeast and human cells. The *in vivo* function of human Gal-Tf indicates that the yeast Golgi is accessible for UDP-Gal and suggests strategies for the construction of yeast strains, in which desired glycoforms of heterologous proteins are produced.

N-Glycosyl chains of glycoproteins can have a great variety of structures in mammalian cells, while they are built relatively simply in lower eukaryotes, such as the yeast *Saccharomyces cerevisiae*. Nevertheless, in both types of cell the dolichol-linked precursor is identical and the selection of asparagine attachment sites proceeds similarly. Yeast core N-glycosyl chains can be extended by the addition of >100 mannose units (1); in contrast, a multitude of enzymes in mammalian cells can process the core to create diverse N-glycosyl chains containing various sugars (2). The complex type of N-glycosyl chains in mammalian cells is synthesized after trimming of the core unit by the sequential addition of GlcNAc, Gal, and NeuAc. Galactose addition in human cells is mediated by UDP-galactose:N-

acetyl-D-glucosaminyl-glycopeptide β -D-galactosyltransferase (EC 2.4.1.38) (Gal-Tf),¹ an enzyme anchored in the Golgi membrane, which uses UDP-Gal as substrate and GlcNAc on glycosyl structures as acceptors (3, 4). In its secreted soluble form, which lacks a membrane anchor region, Gal-Tf functions as lactose synthase, while a third role of Gal-Tf, correlated with its localization on the cell surface, is to mediate adhesion processes (5).

The targeting of Gal-Tf and other glycosyltransferases to the Golgi requires specific protein sequences in these proteins, as well as cellular components (reviewed in Ref. 6). The membrane anchor region of Gal-Tf contains essential sequences for its targeting to the *trans*-Golgi (7–10). Similarly, Golgi localization of yeast glycosyltransferases appears to be mediated by their membrane anchor regions (11, 12). It has been shown that the transmembrane domain of yeast α 1,2-mannosyltransferase (Mnt1p), which extends O-glycosyl chains in the Golgi, is necessary for Golgi localization (11). α 1,3-mannosyltransferase (Mnn1p) contains separable Golgi localization signals within both the transmembrane and luminal domains (12). Recently, it has been shown that the membrane anchor region of a mammalian glycosyltransferase, rat α 2,6-sialyltransferase, is able to target a reporter protein to the yeast Golgi, indicating conservation of targeting mechanisms between lower and higher eukaryotes (13). The transmembrane domain-mediated localization of yeast glycosyltransferases appears distinct from other Golgi proteins, whose targeting depends on Y/F-X-Y/F sequences in their cytoplasmic tails (14).

It has been shown that the production of mammalian glycosyltransferases in heterologous host cells can alter the glycosylation pattern of such cells. Several glycosyltransferases have been isolated by heterologous expression in Chinese hamster ovary cells (reviewed in Refs. 15, 16). In mouse cells transfected with the human gene encoding α 1,2-fucosyltransferase blood group H determinants are synthesized (17). An *Arabidopsis thaliana* mutant defective in N-acetylglucosaminyltransferase I can be complemented by the human cDNA for this enzyme (18). Recently, it has been reported that mammalian glycosyltransferases can be produced in the yeast *S. cerevisiae* in an active form (19–21), but an effect on yeast glycosyl structures was not observed. Such a modifying effect would have been expected only if the respective glycosyltransferase were synthesized at sufficient levels in the proper organelle (such as the Golgi) in yeast cells, on condition that acceptor structures and activated sugars were present (22).

In the present study we demonstrate that a fusion of the

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¹ The abbreviations used are: Gal-Tf, UDP-galactose:N-acetyl-D-glucosaminyl-glycopeptide β -D-galactosyltransferase; ConA, concanavalin A; DAPI, 4',6-diamidino-2-phenylindole; PAGE, polyacrylamide gel electrophoresis; Man-Tf, α 1,2-mannosyltransferase; PNGase F, peptide:N-glycosidase F; RCA, agglutinin RCA₂₀; SD, yeast synthetic minimal medium with dextrose; WGA, wheat germ agglutinin.

membrane anchor region of yeast Mnt1p to soluble human Gal-Tf can be produced at high levels and targeted to the Golgi in yeast cells. We present evidence that the Gal-Tf fusion is enzymatically active and *in vivo* is able to add galactose residues to acceptor structures in an *alg1* mutant. The function of the Gal-Tf fusion in the molecular environment of the yeast Golgi suggests that essential Golgi structures and constituents are conserved between lower and higher eukaryotes and specifically indicates that UDP-Gal is available in the lumen of the yeast Golgi. We propose that the modification of glycosyl structures by the action of heterologous glycosyltransferases may be used as a strategy to construct new yeast host strains that secrete desired glycoforms of applied proteins (23).

EXPERIMENTAL PROCEDURES

Strains and Growth Conditions—*S. cerevisiae* host strains for recombinant vectors were BJ1991 (*MATa ura3-52 leu2 trp1 prb1-1122 pep4-3 gal2*) (24) and ATS594-1B (*MATa ade1 leu2 alg1*). Strain ATS594-1B was constructed by crossing the *alg1* mutant PRY57 (25) to a multiply-marked laboratory strain; among the *alg1 leu2* progeny strain ATS594-1B was chosen, because at the non-permissive temperature (37 °C) it contained high levels of acceptors for *in vitro* galactosylation using human Gal-Tf. Yeast transformants were grown selectively in supplemented SD minimal medium (26). To induce the *GAL10* promoter, cells were grown in SGal-medium containing 2% galactose and 0.2% glucose as carbon sources, as described (19).

Plasmids—The 5' end of the *MNT1* coding region encoding the putative Golgi membrane anchor (27) was isolated first by the polymerase chain reaction using total BJ1991 DNA as template and the two primers 5'-TTACCATGGCCCTCTTTCTCAG-3' and 5'-TCTGGTCTGGCTTCGGAGTCTTCG-3' (the newly added *NcoI* and *AvaII* sites are underlined; the *NcoI* site carries the ATG translational start codon). The amplified fragment was cut with *NcoI* and *AvaII* and ligated to a 602-base pair *BamHI-NcoI* fragment carrying the *GAL10* promoter (derived from p1260/1)² and the large *BamHI-RsrII* fragment of pCT7/J20 (3). The resulting plasmid pGTU1 contained a *MNT1::Gal-Tf* fusion with the following junction sequence (the *MNT1* portion is in italics; numbering corresponds to Mnt1p and Gal-Tf):

5'-GAA GCC AGG ACC GGA GGG GCC-3'
(N) Glu Ala Arg Thr Gly Gly Ala (C)
81 82 *77 78 79 80 81

SEQUENCE 1

The *BamHI-HindIII* fragment carrying the *MNT1::Gal-Tf* expression unit was inserted into the *BamHI* and *HindIII* sites of pJDB207 (28), thereby placing the 3' end of the expression unit next to the 2- μ m *FLP* terminator following the *HindIII* site. The final vector was designated phMGT1 (Fig. 1).

A deleted version of phMGT1 was constructed that only contains residues 1–28 and 75–82 of Mnt1p. First, the *GAL10* promoter was removed from pGTU1 by cutting with *BamHI* and *NcoI*, fill-in of ends with Klenow polymerase, and religation (the *NcoI* site was regenerated during the ligation). The large *EcoRI* fragment (containing vector and N-terminal *MNT1* sequences) and the 1.0-kilobase *EcoRI* fragment (containing *MNT1* and Gal-Tf sequences) of the resulting plasmid pGTUΔ were ligated. In pMNT1ΔRI *MNT1* anchor and Gal-Tf coding regions were fused in-frame. Because of 2 *EcoRI* sites corresponding to amino acids 27/28 and 73/74 of *MNT1* this procedure generated a significant deletion removing 2 amino acids of the membrane anchor (residues 29 and 30) and most residues of the stem region. The *NcoI-HindIII* fusion fragment was joined to the *GAL10* promoter fragment and reinserted into pJDB207, as described above, to construct phMGT3 (Fig. 1).

Cell Fractionations—Crude cell extracts were prepared after cell disruption by glass beads, as described (19). To isolate cell fractions by differential centrifugation, spheroplasted cells were disrupted by a combination of osmotic lysis and mild mechanical breakage, as described (13); consecutive centrifugation at 1000 \times *g*, 10,000 \times *g*, and 100,000 \times *g* yielded pellet and supernatant fractions P1/S1, P2/S2, and P3/S3, respectively. The distribution of enzymatic activities is listed in Table I. Cell fractionation by sucrose gradient centrifugation was performed, as described previously using two methods (13). Briefly, in method A

TABLE I
Distribution of enzyme activities in fractions

Cells were disrupted by mild osmotic lysis (method A) and extracts were fractionated by differential centrifugation. Activities present in the 1000 \times *g* supernatant (S1) were assigned the value 100%.

Enzyme	Activity in fraction				
	S1	S2	P2	S3	P3
	%				
Cytochrome <i>c</i> oxidoreductase	100	43	57	12	31
α -Mannosidase	100	76	27	49	29
Cytochrome <i>c</i> oxidase	100	32	68	0	32
Man-Tf	100	55	45	15	40
Gal-Tf (phMGT1)	100	44	56	3	41
Gal-Tf (phMGT3)	100	41	59	9	32

spheroplasted cells were disrupted and fractionated as for differential centrifugation; organelles in the pellet fractions P2 and P3 were allowed to float up from the bottom of a discontinuous sucrose gradient (20–55%); Table I demonstrates that over 90% of the Gal-Tf activity present in the crude extract is present in fractions P2 and P3. In method B spheroplasts were disrupted by glass beads and organelles in the crude extract distributed from the top of the sucrose gradient (13).

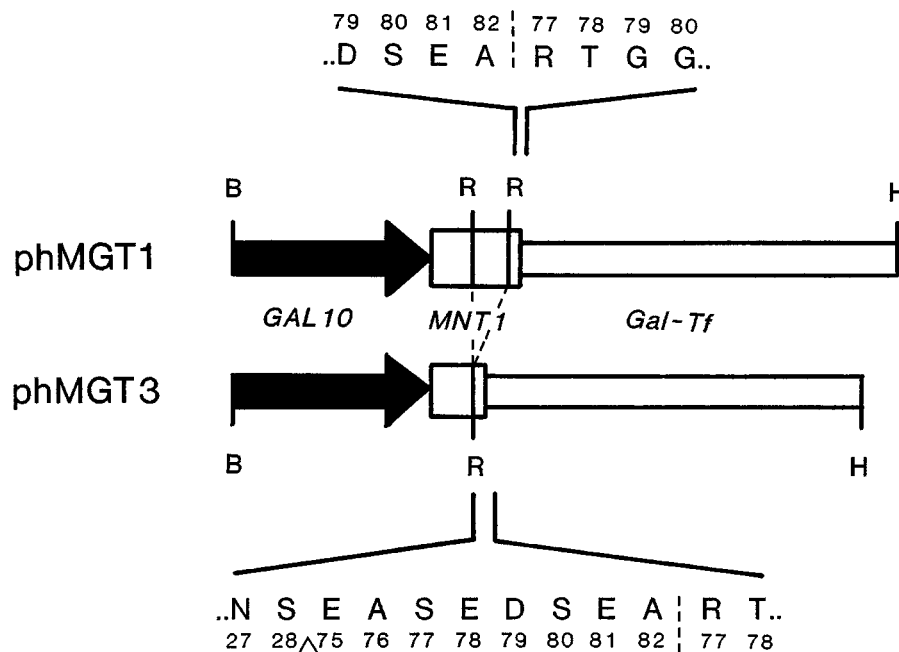
Protease Protection Assay—To examine protease accessibility, cells (100 OD_{600 nm} units) were spheroplasted and broken as in method A, except that protease inhibitors were omitted. The cell extract was centrifuged at 3000 \times *g* and the supernatant was used for protease protection. The assay contained, in 35 μ l of total volume, 25 μ l of supernatant, 1 μ l of proteinase K (Merck) (1 mg/ml in 50 mM Tris-HCl, pH 7.4, 100 mM NaCl, 250 mM sucrose) and, if applicable, 4 μ l of Triton X-100 (1%). The assay was incubated 15 min on ice, after which 1 μ l of phenylmethylsulfonyl fluoride (40 mg/ml in EtOH) and 35 μ l of 2 \times Laemmli sample buffer was added. The sample was heated (95 °C/10 min) and proteins were separated by SDS-polyacrylamide gel electrophoresis (PAGE) (10% acrylamide, 0.25% *N,N'*-methylene bisacrylamide). An immunoblot was performed using monoclonal anti-Gal-Tf (MAB8628) (29) as first antibody (dilution 1:1000) and alkaline phosphatase-coupled rabbit anti-mouse antibody (Jackson ImmunoResearch; dilution 1:5000) as secondary antibody.

Immuno/Lectin Precipitations—Immunoprecipitations of ³⁵S-labeled crude extracts were performed essentially as described (13). In sequential precipitations 7.5 μ l of anti-Gal-Tf (MAB8628) (29), 5 μ l of anti- α 1,6-mannose, or 5 μ l of anti- α 1,3-mannose antibodies were used (13); to precipitate MAB8628 protein complexes we used a complex of rabbit anti-mouse IgG (Sigma) to protein A-Sepharose, as described (30). Alternatively, 35 μ l of 20% concanavalin A (ConA)-Sepharose 4B (Sigma) was added to precipitate mannosylated proteins.

To monitor the *in vivo* activity of Gal-Tf, transformants of strain ATS594-1B were grown at 26 °C to an OD_{600 nm} of 0.5–0.8 in low sulfate medium containing 2% galactose and 0.2% glucose (13). 10 OD_{600 nm} units were harvested and resuspended in 1 ml of low sulfate medium lacking glucose and containing 0.24% bovine serum albumin. After preincubation at 37 °C, 26 μ l of Tran³⁵S-label (ICN Biomedicals, Inc.) (300 μ Ci) were added and the sample was incubated further at 37 °C for 60 min. 20 μ l of 50 \times chase solution (50 mM (NH₄)₂SO₄, 250 mM L-methionine, 50 mM L-cysteine) was added and the incubation was continued for 15 min. Cells were harvested by centrifugation (4000 rpm/5 min), washed twice with 2 ml of ice-cold 10 mM Na₂S₂O₄, 10 mM dithiothreitol and resuspended in 450 μ l of LIP buffer (50 mM Tris-HCl, pH 7.4, 1% Triton X-100, 150 mM NaCl, 5 mM EDTA). Cells were frozen at this stage at –80 °C prior to further processing. Phenylmethylsulfonyl fluoride was added to 1 mM and cells were thawed on ice and disrupted with an equal volume of glass beads on a Braun homogenizer (2 \times 2 min). Following inactivation of Gal-Tf at 95 °C (5 min) the extracts were placed on ice and centrifuged 2 min at 5000 rpm. The supernatant was transferred to a new vial, 50% (NH₄)₂SO₄ was added to a total volume of 1.5 ml and allowed to stand on ice for a minimum of 3 h. The suspension was centrifuged for 5 min at 14,000 rpm (4 °C), after which the supernatant was discarded and the protein pellet was washed with 500 μ l of ice-cold 25% (NH₄)₂SO₄. The pellet was solubilized in 50–100 μ l of 0.2% Triton X-100 by vortexing and LIP buffer was added to 1.5 ml. The solution was precleared by the addition of 30 μ l of 50% Sephacryl S-200 (Pharmacia) and mild agitation at 4 °C for 1–2 h, followed by centrifugation at 14,000 rpm (2 min). The supernatant was divided in 3 portions of 500 μ l, to which were added either 40 μ l of 20% ConA-Sepharose 4B (Sigma), or 40 μ l of 50% agarose-coupled wheat germ agglutinin (WGA) (Sigma), or 40 μ l of 50% agarose-coupled ag-

² J. F. Ernst, unpublished data.

FIG. 1. Structures of MNT1/Gal-Tf expression units. The MNT1 and Gal-Tf gene segments are indicated (open boxes); the GAL10 promoter is represented by the arrow. Sequences at the fusion junctions (dashed line) of the encoded proteins are shown along with residue numbers of the original proteins. The position of the deletion in the phMGT3-encoded fusion is indicated. B, BamHI; R, EcoRI; H, HindIII.



glutinin RCA₁₂₀ (RCA) (Sigma). Following mild agitation overnight at 4 °C the suspensions were centrifuged (14,000 rpm/4 °C), the supernatants discarded, and the pellets were washed twice with LIP buffer and twice with phosphate-buffered saline. Pellets were resuspended in Laemmli sample buffer and proteins were separated by SDS-PAGE.

Enzyme Assays—The transfer of [¹⁴C]galactose from UDP-[¹⁴C]Gal to ovalbumin was determined according to Verdon and Berger (31). The assay contained in a total volume of 50 μ l: 50 mM HEPES, pH 7.35, 10 mM MnCl₂, 1.5 mg of ovalbumin, 54 mM NaCl, 5 μ l of UDP-[¹⁴C]galactose (2.5 μ mol; 25 nCi), and 5 μ l of sample. The assay was incubated for 60 min at 30 °C and the reaction was stopped by the addition of ice-cold 2.5% phosphotungstic acid (w/v) in 1 M HCl. Unincorporated UDP-[¹⁴C]Gal was separated by filtration through Whatman GF/C glass fiber filters. The filters were washed once with 2.5% phosphotungstic acid (w/v) and then rinsed with ice-cold ethanol. The filters were dried at 60 °C and radioactivity was determined in a scintillation counter. 285 μ g of purified human Gal-Tf (Boehringer) has an enzymatic activity of 1 unit, if GlcNAc is used as acceptor (in the absence of α -lactalbumin); with ovalbumin as acceptor we determined that this amount of Gal-Tf has an enzymatic activity of 0.2 units (specific activity 0.8 units/mg) (19). The assays for NADPH-dependent cytochrome *c* oxidoreductase (ER-marker), cytochrome *c* oxidase (mitochondrial marker), α 1,2-mannosyltransferase (Golgi marker), and α -mannosidase (vacuolar marker) were determined as described (13).

Immunofluorescence Microscopy—Cells were prepared for immunofluorescence microscopy essentially as described (32), with the following modifications: spheroplasts were prepared by digesting 4 OD_{600 nm} units of cells in 1 ml of buffer A (50 mM HEPES, 10 mM Na₂S₂O₈, 1.2 M sorbitol, pH 7.5) containing 10 mM dithiothreitol, using 30 μ l of glucuronidase (Sigma) and 10 μ l of zymolase T100 (Seikagaku Kogyo Co. Ltd.) for 30 min at 37 °C; following spheroplast formation additional permeabilization was achieved by a 5-min incubation with 0.1% Triton X-100. Anti-Gal-Tf (monoclonal 8628, Ref. 29) was used as primary antibody, which was diluted 1:1000 in phosphate-buffered saline containing 0.1% bovine serum albumin (essentially globulin-free; Sigma). As secondary antibody goat anti-mouse antibody, coupled to dichlorotriazinyl amino fluorescein, was used according to the recommendation of the manufacturer (Dianova). DNA was stained using 4',6-diamidino-2-phenylindole (DAPI) as described (32).

Other Methods—ATPase in gradient fractions was determined by immunoblotting using anti-ATPase antibody; immunoblots were evaluated by densitometry using a Molecular Dynamics laser densitometer (13). Protein concentrations were determined using the Bio-Rad reagent with bovine serum albumin as standard. Digestions by peptide: N-glycosidase F (PNGase F) were performed according to the protocol of the manufacturer (New England Biolabs). The sucrose concentrations in the gradient fractions were determined using the refractive index.

RESULTS

Construction of MNT1::Gal-Tf Gene Fusions—To produce enzymatically active human Gal-Tf in the yeast Golgi we constructed a gene fusion encoding 82 N-terminal residues of yeast Mnt1p (27) fused to residues 77–400 of human Gal-Tf (3) (Fig. 1). The Mnt1p portion contains the membrane anchor region, which has been involved in Golgi targeting of Mnt1p (11); the Gal-Tf portion encodes the soluble part of Gal-Tf, but lacks targeting sequences. The expression unit was placed under transcriptional control of the GAL10 promoter, inserted in a yeast transformation vector (resulting plasmid phMGT1), and transformed in a *S. cerevisiae* strain. A derivative expression plasmid (phMGT3) encoding a shortened fusion protein containing only the N-terminal residues 1–28 and 75–82 of Mnt1p was constructed and transformed (Fig. 1).

Production of Gal-Tf—To demonstrate synthesis of Gal-Tf in transformants carrying phMGT1 or phMGT3 we separated crude extracts by SDS-PAGE and demonstrated Gal-Tf-related proteins by immunoblotting using a monoclonal anti-Gal-Tf antibody (29) (Fig. 2A). Crude extracts of phMGT1 transformants contained an anti-Gal-Tf-reactive protein of about 50 kDa, while the Gal-Tf-species in phMGT3 transformants had a size of about 45 kDa (Fig. 2A, lanes 1 and 2). Control transformants carrying the basic vector pJDB207 did not contain a Gal-Tf protein (Fig. 2A, lane 3).

Gal-Tf contains a single potential site for N-glycosylation, which is glycosylated in human cells (33). To test the glycosylation status of the yeast-produced Gal-Tf fusion we treated immunoprecipitates of ³⁵S-labeled transformants with PNGase F. Gal-Tf was immunoprecipitated using an anti-Gal-Tf antibody; the precipitates were separated by SDS-PAGE and gel patterns were visualized by autoradiography (Fig. 2B). PNGase F-treated Mnt1/Gal-Tf migrates slightly faster than untreated Mnt1/Gal-Tf, an effect that is especially apparent for the phMGT3-encoded Gal-Tf (Fig. 2B, compare lanes 4, 5 and 6, 7). We estimate that N-glycosylation increases the molecular mass of the yeast-produced Mnt1/Gal-Tf fusion protein by about 3 kDa.

The small size of its N-glycosyl chain was consistent with only core N-glycosylation of the Gal-Tf fusion. To test if the fusion protein nevertheless had acquired typical Golgi modifi-

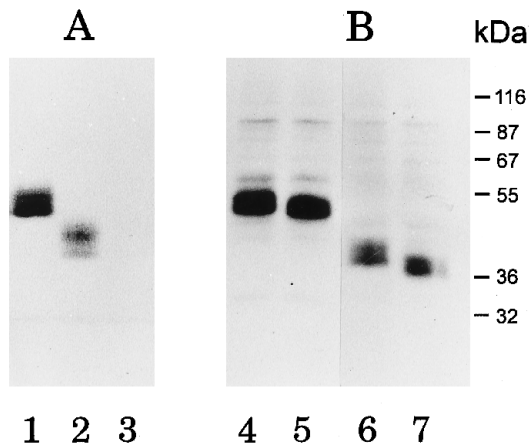


FIG. 2. Production of Mnt1/Gal-Tf fusions by yeast transformants. A, extracts of transformants carrying phMGT1 (lane 1), phMGT3 (lane 2), and pJDB207 (lane 3) were analyzed by immunoblotting using a monoclonal anti-Gal-Tf antibody. B, transformants were labeled by [35 S]methionine and proteins in cell extracts were immunoprecipitated using anti-Gal-Tf antibody. Immunoprecipitates were analyzed by SDS-PAGE followed by autoradiography. Extracts of a phMGT1 transformant (lanes 4 and 5) and of a phMGT3 transformant (lanes 6 and 7) are shown; samples loaded in lanes 5 and 7 had been treated by PNGase F. The apparent molecular masses of prestained standard proteins (Sigma) are indicated.

cations, sequential immunoprecipitations were carried out by first using anti-Gal-Tf antibody (29), followed by either anti- α 1,6-mannose or anti- α 1,3-mannose antibodies; in addition, in the second step aliquots were immunoprecipitated with concanavalin A or, as a control, with anti-Gal-Tf antibody. Similar results were obtained for the phMGT1- and phMGT3-encoded fusion proteins (Fig. 3). The anti- α 1,6-mannose antibody and concanavalin A were similarly efficient as the anti-Gal-Tf antibody to precipitate the Gal-Tf fusions (Fig. 3, lanes 2, 3, 5 or lanes 7, 8, 10); however, no immunoprecipitation occurred with the anti- α 1,3-mannose antibody. These results indicate that the Gal-Tf fusion proteins have quantitatively obtained the α 1,6-mannose modification in the Golgi; the absence of α 1,3-modification indicates that the fusion proteins have not reached distal Golgi compartments.

If the Mnt1/Gal-Tf fusions had inserted into yeast organelle membranes as the authentic Mnt1 and Gal-Tf proteins (4, 11, 34), which are type II membrane proteins, it could be expected that they are refractory to the action of proteases. To test this notion crude extracts of phMGT1 and phMGT3 transformants were treated with proteinase K in the presence and absence of Triton X-100; subsequent to the treatments the Gal-Tf fusion protein was analyzed on immunoblots using the anti-Gal-Tf antibody. It is demonstrated in Fig. 4 that the phMGT1- and phMGT3-encoded fusion proteins showed identical properties in this assay: the Gal-Tf fusion protein was only stable in the presence of protease, if no detergent was added (Fig. 4, lanes 3 and 7), while degradation occurred in its presence (Fig. 4, lanes 4 and 8). The shortened Gal-Tf fusion encoded by phMGT3 appeared somewhat more resistant in this assay, suggesting that the deleted region compared to phMGT1 contains sequences especially sensitive to proteolysis. The protease protection experiments clearly indicate that the Mnt1/Gal-Tf fusion proteins reside in a closed vesicular compartment.

Golgi Localization of Gal-Tf Fusions—Although the presence of α 1,6-determinants on the Gal-Tf fusions strongly suggested a Golgi localization, it could not be excluded that these proteins only temporarily had reached a Golgi compartment prior to being retrieved to a preceding secretion compartment (30). To verify the final localization of the Gal-Tf fusions we performed

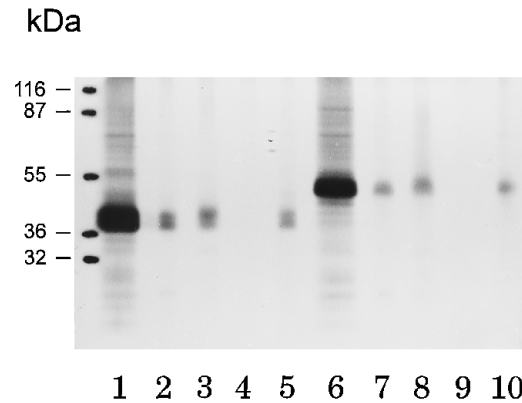


FIG. 3. Golgi modification of Mnt1/Gal-Tf fusions. Immunoblotting analyses of yeast extracts were performed as in Fig. 2B. Immunoprecipitates obtained with anti-Gal-Tf antibody (lanes 1 and 6) were solubilized, and aliquots were again immunoprecipitated with anti-Gal-Tf antibody (lanes 2 and 7), anti- α 1,6-mannose antibody (lanes 3 and 8), anti- α 1,3-mannose antibody (lanes 4 and 9), or concanavalin A (lanes 5 and 10). Extracts of the phMGT3 transformant (lanes 1–5) and the phMGT1 transformant (lanes 6–10) were analyzed.

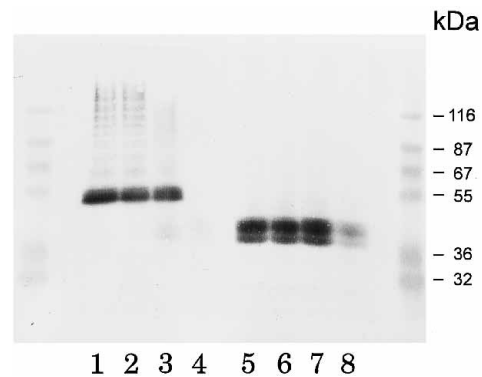


FIG. 4. Protease protection of Mnt1/Gal-Tf fusions. Extracts of a phMGT1 transformant (lanes 1–4) and a phMGT3 transformant (lanes 5–8) were not pretreated (lanes 1, 2, 5, and 6) or pretreated with proteinase K (lanes 3, 4, 7, and 8) in the absence (lanes 1, 3, 5, and 7) or presence of Triton X-100 (lanes 2, 4, 6, and 8).

sucrose gradients and examined colocalization with organelle markers. We used two methods of cell breakage prior to gradient fractionation, either using a combination of osmotic lysis and mild mechanical disruption (method A), or breakage by glass beads (method B) (13). The results of the gradient fractionations of phMGT1 transformants are shown in Fig. 5 (method A) and Fig. 6 (method B). In Fig. 5C it is shown that the distribution of Mnt1/Gal-Tf closely parallels the distribution of the enzymatic activity of Man-Tf with a peak in fraction 8; only the higher density fractions 10–13 contain more of the fusion protein than expected from the Man-Tf activity. The endoplasmic reticulum marker NADPH-dependent cytochrome *c* oxidoreductase and the mitochondrial marker cytochrome *c* oxidase peak in fractions 11 and 12, respectively (Fig. 5B), while the vacuolar marker α -mannosidase fractionates at the bottom of the gradient (Fig. 5B). Fractionation by method B is disadvantageous in that the glass bead breakage disrupts large organelles, such as the vacuole; on the other hand, vacuolar and plasma membrane markers can be well resolved (Fig. 6B). Mnt1/Gal-Tf again comigrates with Man-Tf in this gradient (Fig. 6C) and clearly does not distribute with the plasma membrane ATPase. In separate analogous localization experiments almost identical results were obtained for transformants carrying phMGT3 (data not shown).

To confirm Golgi localization of the Mnt1/Gal-Tf fusion, immunocytological localization experiments using the monoclonal

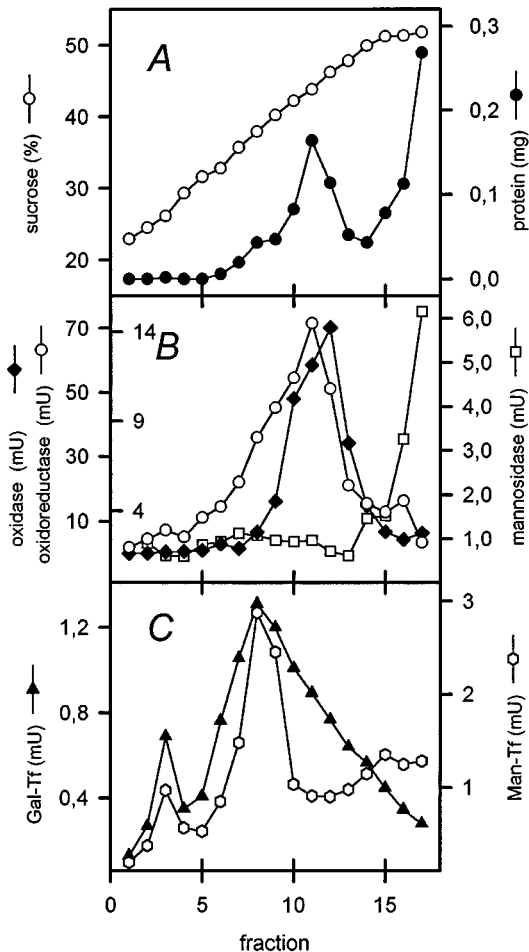


FIG. 5. Mnt1/Gal-Tf and marker proteins in cell fractions obtained by sucrose gradient centrifugation. Organelles of a phMGT1 transformant, prepared by method A (see "Experimental Procedures"), were separated on a sucrose density gradient, and gradient fractions were analyzed for the presence of Mnt1/Gal-Tf and marker proteins by enzymatic activity tests. Gal-Tf, Gal-Tf activity of Mnt1/Gal-Tf fusion; oxidoreductase, NADPH-dependent cytochrome *c* oxidoreductase; oxidase, cytochrome *c* oxidase; mannosidase, α -mannosidase.

anti-Gal-Tf antibody were carried out. phMGT1-transformant cells, but not control transformants carrying pJDB207, displayed a punctuated immunofluorescent staining characteristic of the yeast Golgi (11). No fluorescence was detected in the vacuole, which was clearly visible by differential interference contrast microscopy or in association with the nucleus, which was stained by DAPI (Fig. 7). Thus, the fractionation and immunocytological localization experiments indicate that the Mnt1p/Gal-Tf fusions encoded by the phMGT vectors are located in the Golgi and not in other cellular organelles.

In Vitro Gal-Tf Activity—In a previous comparative study on production of human Gal-Tf fused to various secretion leaders in yeast we found that based on enzymatic activity the Mnt1/Gal-Tf fusion (encoded by phMGT1) was synthesized at high levels intracellularly (19) amounting to $373.3 \mu\text{g}/3.5 \times 10^{10}$ cells (cells contained in 1 liter of cell culture at $\text{OD}_{600 \text{ nm}} = 1$). Using the monoclonal anti-Gal-Tf antibody MAb8628 (29) in immunoblot comparisons to purified human Gal-Tf the absolute Mnt1/Gal-Tf protein level in transformants was directly determined as $677 \mu\text{g}/3.5 \times 10^{10}$ cells. Enzymatic activity tests were performed, in which equal amounts of the yeast-produced Mnt1/Gal-Tf fusion (in the phMGT1 transformant) and authentic human Gal-Tf were directly compared (Fig. 8). Using ovalbumin as acceptor we obtained similar K_m values for UDP-

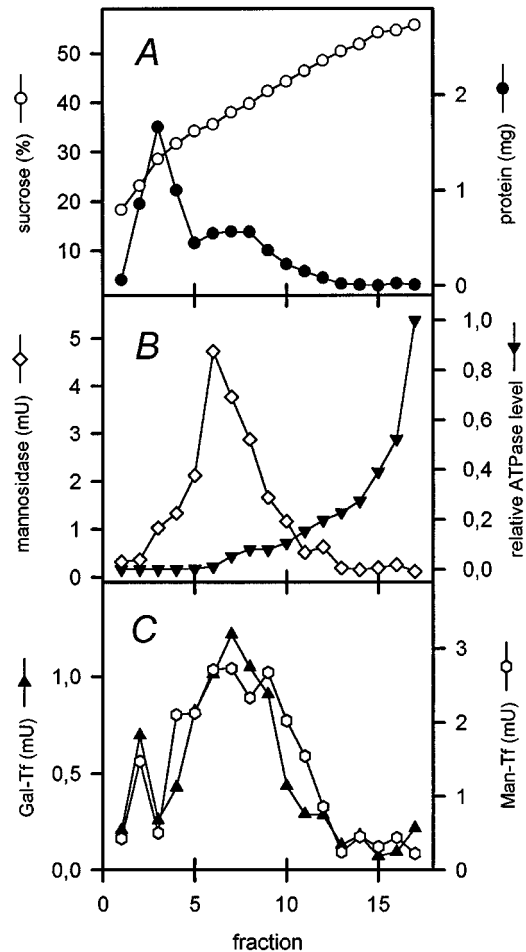


FIG. 6. Mnt1/Gal-Tf and marker proteins in cell fractions obtained by sucrose gradient centrifugation. Cell extracts of a phMGT1 transformant, prepared by method B (see "Experimental Procedures"), were separated on a sucrose density gradient and analyzed as described in the legend to Fig. 5. The relative ATPase protein level is indicated (the maximal level in fraction 17 was arbitrarily assigned the value 1.0).

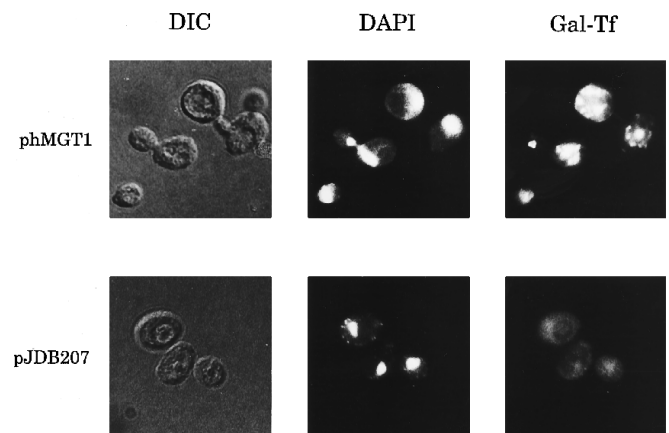


FIG. 7. Immunocytological localization of Gal-Tf. Transformants carrying phMGT1 or pJDB207 were treated with anti-Gal-Tf antibody, which was reacted with dichlorotriazinyl aminofluorescein-coupled anti-mouse antibody; in addition, cells were treated with DAPI. The appearance of cells as detected by differential-interference-contrast microscopy (DIC), DAPI-fluorescence, and indirect immunofluorescence (for Gal-Tf) was examined.

Gal: $44 \mu\text{M}$ for the Mnt1/Gal-Tf fusion and $45 \mu\text{M}$ for the human soluble protein. In contrast, the V_{max} values were $0.184 \mu\text{mol}/\text{min}/\text{mg}$ for Mnt1/Gal-Tf and $0.8 \mu\text{mol}/\text{min}/\text{mg}$ for human Gal-

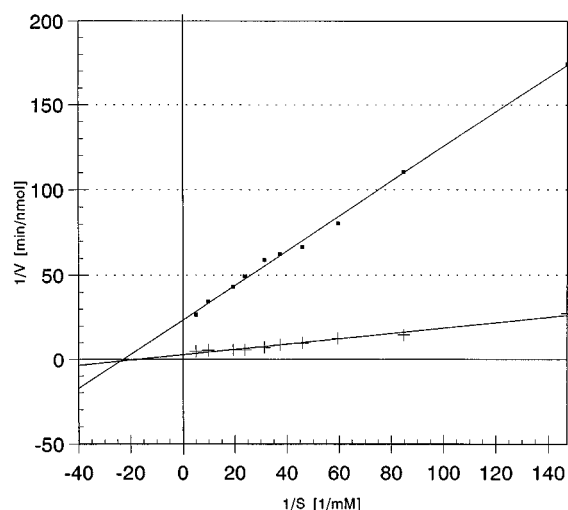


FIG. 8. ***In vitro* Gal-Tf activity.** The enzymatic activity of 0.25 μ g of Mnt1/Gal-Tf in cell extracts of a BJ1991[phMGT1] transformant (■) and of soluble human Gal-Tf (+) were determined with ovalbumin as acceptor protein.

Tf. The similarity in K_m values indicates that the fusion protein is produced in a fully functional form in the yeast Golgi; possibly, the attachment of the Mnt1p membrane anchor region is responsible for the lowering of the V_{max} value. The transfer of galactose to ovalbumin in the *in vitro* assay could be blocked by an excess of free GlcNAc, as expected (Table II).

In Vivo Gal-Tf Activity—Because the previous experiments had indicated that the Mnt1/Gal-Tf fusion is inserted in the yeast Golgi in an active form we speculated that this enzyme would be active *in vivo*, provided that UDP-Gal and an acceptor glycosyl chain were present. During growth on galactose, UDP-Gal is synthesized and the *GAL10* promoter driving transcription of the *MNT1::Gal-Tf* gene fusions in transformants is induced. Yeast *alg1* mutants at the non-permissive temperature are known to transfer incomplete glycosyl chains, consisting of GlcNAc- β 1,4-GlcNAc, to Asn residues of secreted proteins (25). Therefore, we transformed plasmids phMGT1/3 and control plasmid pJDB207 into the yeast *alg1* host strain ATS594-1B and labeled transformant proteins with 35 S at the non-permissive temperature, using galactose as a carbon source. We then used immobilized sugar- and linkage-specific lectins to precipitate proteins carrying specific sugars on the newly synthesized glycosyl chains: ConA reacts with α -linked mannose, WGA reacts with GlcNAc- β 1,4-GlcNAc, and RCA detects β -linked terminal galactose.

In transformants carrying control vector pJDB207 several proteins were precipitated with ConA and WGA (Fig. 9, lanes 1 and 2), indicating mannose and GlcNAc residues; the high level of mannosylated proteins suggests that the *alg1* mutation in strain ATS594-1B is leaky at the non-permissive temperature (25). No proteins could be precipitated with lectin RCA, demonstrating the absence of terminal galactose in ATS594-1B[pJDB207] transformants (Fig. 9, lane 3). In contrast, several proteins could be detected in transformants carrying plasmid phMGT1 (Fig. 9, lane 6) (similar results were obtained for the phMGT3 transformant). Although in this experiment Gal-Tf was heat-inactivated, we considered the possibility that GlcNAc-carrying proteins only became galactosylated because of the liberation of Gal-Tf and yeast proteins during cell breakage. In a control experiment we added 100 mM GlcNAc before cell breakage, whose presence competes with the transfer of galactose to ovalbumin in the *in vitro* Gal-Tf assay (Table II). Since proteins still became efficiently galactosylated after this treatment (Fig. 10) this process must have occurred in the

TABLE II
Inhibition of Gal-Tf activity by GlcNAc
In vitro Gal-Tf activity (ovalbumin as acceptor) in yeast extracts without or with 100 mM GlcNAc. Units present in membrane extracts (method A) of 100 OD_{600 nm} units of cells (6.6 mg of protein) are listed.

Plasmid in transformant	GlcNAc	Gal-Tf
		milliunits
pJDB207	—	0
pJDB207	+	0
phMGT1	—	6.61
phMGT1	+	0.28
phMGT3	—	5.65
phMGT3	+	0.29

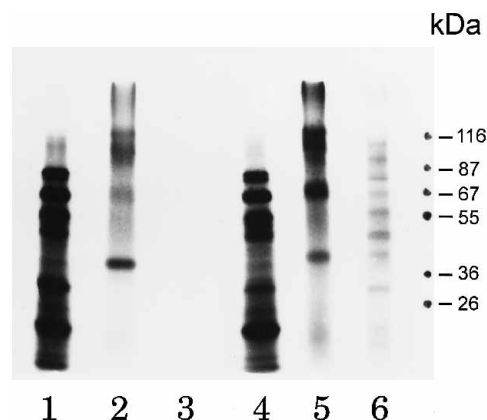


FIG. 9. ***In vivo* Gal-Tf activity.** The *alg1* strain ATS594-1B carrying control vector pJDB207 (lanes 1–3) or phMGT1 (lanes 4–6) was 35 S-labeled at the non-permissive temperature. Glycosylated proteins were precipitated with immobilized lectins ConA (lanes 1 and 4), WGA (lanes 2 and 5), or RCA (lanes 3 and 6) and separated by SDS-PAGE, followed by autoradiography.

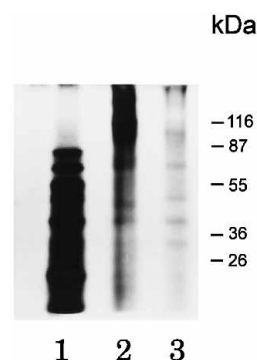


FIG. 10. ***In vivo* Gal-Tf activity in the presence of GlcNAc.** Protein glycosylation of strain ATS594-1B[phMGT1] was determined essentially as described in the legend to Fig. 9, but by using cells broken in the presence of 100 mM GlcNAc. For lectin-precipitations ConA (lane 1), WGA (lane 2), and RCA (lane 3) were used.

intact cell, *i.e.* during the transit of proteins via the Golgi. These results show that expression of the *MNT1::Gal-Tf* gene fusion is able to allow the galactosylation of a number of secreted yeast proteins. The presence of non-galactosylated proteins carrying GlcNAc residues (Fig. 9, compare lanes 5 and 6) may be due to proteins, whose transit from the endoplasmic reticulum to the Golgi does not occur in the absence of normal core glycosylation.

DISCUSSION

We report here that a mammalian glycosyltransferase, human Gal-Tf, can be targeted to the yeast Golgi, where it is inserted in authentic orientation and attains an active conformation. We present evidence that the yeast-produced Gal-Tf is

able to add galactose residues to the truncated glycosyl chains of *alg1* mutants. These results have implications regarding the basic mechanisms of targeting and function of glycosyltransferases, as well as for the use of yeast as a host for the production of heterologous proteins.

We previously found that the membrane anchor region of rat $\alpha 2,6$ -sialyltransferase is able to direct the localization of a reporter protein to the yeast Golgi (13). Likewise, a fusion of the human Gal-Tf membrane anchor region to invertase was targeted to the yeast Golgi, although production levels were low.³ Initial experiments on expression of the gene encoding full-length human Gal-Tf in yeast, however, were unsuccessful, because Gal-Tf was not synthesized in spite of a considerable transcript level (19). Therefore, we chose to express a gene fusion encoding the membrane anchor region of a yeast glycosyltransferase, Mnt1p, fused to the soluble form of Gal-Tf (plasmid pHMGT1). This gene fusion is expressed in yeast at high transcript levels and leads to high intracellular levels of Gal-Tf enzymatic activity (19). We had speculated that high transcript levels may be caused by a "downstream activating sequence" present in *MNT1*; if such a sequence exists it does not appear to be situated in the region encoding residues 29–74, because a deletion of this region (in pHMGT3) does not affect levels of Gal-Tf synthesis. The membrane anchor region of Mnt1p has been shown previously to direct a reporter enzyme to the yeast Golgi (11). In the present study we confirm and extend these results by demonstrating that the first 28 N-terminal residues of Mnt1p encompassing the cytoplasmic tail, the membrane anchor region plus 8 non-contiguous residues of the stem region are sufficient to achieve Golgi localization. This result indicates that luminal sequences adjacent to the transmembrane region, as in Mnn1p (12), do not contribute to Golgi targeting of Mnt1p. Golgi localization was proven by the presence of $\alpha 1,6$ -mannose determinants on Gal-Tf, a modification occurring in the early Golgi (1). Because some secreted proteins only temporarily reach the Golgi before being retrieved to a preceding compartment (30) we demonstrated in two different types of sucrose gradients that Gal-Tf comigrates with Golgi marker proteins, but not with markers specific for other organelles. Since Gal-Tf did not receive $\alpha 1,3$ -mannose determinants it appears that its final localization is in the *cis*- or *medial*-Golgi, but not the *trans*-Golgi (1). Thus, besides the already known targeting mechanism using Y/F-X-Y/F sequences in the cytoplasmic tail (14), yeast appears to use a second mechanism independent of such sequences, such as used by Mnt1p, Mnn1p, and the heterologous sialyltransferase membrane anchor (11–13).

The function of a mammalian membrane anchor region and, as we report here, the function of a human glycosyltransferase in the molecular environment of the yeast Golgi demonstrates that essential features affecting the localization and function of glycosyltransferases are conserved between lower and higher eukaryotes. Thus, the function of Gal-Tf in yeast does not require auxiliary proteins present only in human cells. In the *in vitro* assay K_m values for UDP-Gal of yeast-produced and human Gal-Tf were identical; the lowered V_{max} values may be due to the lowered reactivity of the membrane anchor-coupled (yeast) Gal-Tf compared to free (human) Gal-Tf with respect to the ovalbumin acceptor in this assay. The observed *in vivo* activity of Gal-Tf was unexpected in spite of the presence of UDP-Gal and the *alg1* acceptor structures in the transformants, because yeast does not add galactose to proteins and a transport system to import UDP-Gal in the Golgi lumen is not known and does not seem necessary (22). Our results imply

that UDP-Gal is present in the lumen of the Golgi; this could occur by the action of a UDP-Gal transporter in the Golgi membrane, which functions analogous to the GDP-Man carrier as an antiport system exchanging luminal UMP with cytoplasmic UDP-Gal. The latter mechanism appears possible, because a UDPase activity that generates UMP from UDP, is known to be present in yeast (35). However, the existence of a Golgi UDP-Gal import system remains to be demonstrated directly; such studies also may reveal, if UDP-Gal co-utilizes existing transporters for GDP-Man or, possibly, UDP-Glc(NAc).

The use of a *alg1* mutant allowed the demonstration of *in vivo* activity of Gal-Tf by providing glycosyl acceptors of the structure GlcNAc- $\beta 1,4$ -GlcNAc (25). The temperature sensitivity of this strain was not remedied by Golgi galactosylation, most likely because the short *N*-glycosyl chains prevent transit of those proteins from the endoplasmic reticulum to the Golgi, whose folding depends on more extended glycosyl chains. However, many *alg* or *mnn* mutants are known, in which a variety of incomplete glycosyl chains are produced; based on the principle shown in this study such mutants may be used as hosts for the synthesis of heterologous glycosyltransferases in an attempt to alter glycosyl structures. It also appears feasible to simultaneously produce more than one glycosyltransferases in one strain, such as to generate more complex glycoforms *in vivo*. It will be an even greater challenge to add synthesis reactions for activated sugars not present in yeast, such as for the synthesis of CMP-NeuAc. We anticipate that reconstitution of glycosylation pathways in yeast will on the one hand help clarify the biology of mammalian glycosylation reactions. On the other hand new host strains will be generated that will allow the production of heterologous proteins, whose glycosyl chains are modified by the addition of, for example, terminal Gal residues. These could be initially further modified *in vitro* by the addition of sialic acid (36) with the goal to shield mannose residues that are targets for mannose-binding proteins and specific antibodies (23).

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