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Uridine Diphosphate–Glucose Transport into the Endoplasmic Reticulum of *Saccharomyces cerevisiae*: In Vivo and In Vitro Evidence

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It has been proposed that synthesis of β-1,6-glucan, one of *Saccharomyces cerevisiae* cell wall components, is initiated by a uridine diphosphate (UDP)-glucose–dependent reaction in the lumen of the endoplasmic reticulum (ER). Because this sugar nucleotide is not synthesized in the lumen of the ER, we have examined whether or not UDP–glucose can be transported across the ER membrane. We have detected transport of this sugar nucleotide into the ER in vivo and into ER–containing microsomes *in vitro*. Experiments with ER-containing microsomes showed that transport of UDP–glucose was temperature dependent and saturable with an apparent K_m of 46 μM and a V_{max} of 200 pmol/mg protein/3 min. Transport was substrate specific because UDP–*N*-acetylglucosamine did not enter these vesicles. Demonstration of UDP–glucose transport into the ER lumen in vivo was accomplished by functional expression of *Schizosaccharomyces pombe* UDP–glucose:glycoprotein glucosyltransferase (GT) in *S. cerevisiae*, which is devoid of this activity. Monoglucosylated protein-linked oligosaccharides were detected in *alg6* or *alg5* mutant cells, which transfer $Man_9GlcNAc_2$ to protein; glucosylation was dependent on the inhibition of glucosidase II or the disruption of the gene encoding this enzyme. Although *S. cerevisiae* lacks GT, it contains Kre5p, a protein with significant homology and the same size and subcellular location as GT. Deletion mutants, k*re5*Δ, lack cell wall β-1,6 glucan and grow very slowly. Expression of *S. pombe* GT in k*re5*Δ mutants did not complement the slow-growth phenotype, indicating that both proteins have different functions in spite of their similarities.

INTRODUCTION

There are four major polysaccharide constituents in the *Saccharomyces cerevisiae* cell wall: mannan, chitin, and β-1,6- and β-1,3-glucans (Cid *et al.*, 1995). Whereas synthesis of *mannan* is initiated in the endoplasmic reticulum (ER)[1] by transfer of $Glc_3Man_9GlcNAc_2$ to a protein core and continues by the transfer of additional mannose units in the Golgi, both chitin and β-1,3-glucan are synthesized at the plasma membrane (Cid *et al.*, 1995). However, it is not clear where the synthesis of β-1,6-glucan is initiated and/or completed, although the intracellular presence of β-1,6-glucan has been detected by immunogold labeling (Horrisberg and Clerk, 1987). Mutants defective in the biosynthesis of β-1,6-glucan (kre) have been identified based on their resistance to K_1 killer toxin. Several *KRE* gene products implicated in β-1,6-glucan biosynthesis reside along the secretory pathway. Kre5p and Cwh41p are located in the ER, Kre6p and Skn1p are located in the Golgi apparatus, Kre1p appears to be in the plasma membrane (Boone *et al.* 1990; Meaden *et al.*, 1990; Jiang *et al.*, 1996), and Kre9p is also located in the

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[1] Abbreviations used: DNJ, 1-deoxynojirimycin; Endo H, endo-β-*N*-acetylglucosaminidase H; ER, endoplasmic reticulum; GII, glucosidase II; GT, UDP–glucose:glycoprotein glucosyltransferase; UDP–GlcNac, uridine diphosphate *N*-acetyl glucosamine.

secretory pathway (Brown and Bussey, 1993). It is not known which of these proteins are directly implicated in β-1,6-glucan synthesis, but some proteins have been shown to influence it indirectly. Cells lacking either ER glucosidase I (Cwh41p) or glucosidase II (GII) were found to display a severe reduction in β-1,6-glucan content (Jiang *et al.*, 1996; Simons *et al.*, 1998). The glucosidases are required for removal of the glucose residues from protein-linked $Glc_3Man_9GlcNAc_2$, during processing of N-glycans. It was shown that it is the retention of the glucose units on the N-linked oligosaccharides that affects the β-1,6-glucan synthesis in glucosidase mutants. Double mutants that do not add glucose to the lipid-linked oligosaccharide precursor have wild-type levels of β-1,6-glucan (Abeijón and Chen, 1998; Shahinian *et al.*, 1998). Kre5p encodes a luminal ER protein of 150 kDa that is essential for *S. cerevisiae* viability in certain genetic backgrounds. However, null mutants that are viable have no detectable β-1,6-glucan polymer, show aberrant morphology, are unable to retain cell wall mannoproteins in their cell wall, and have extremely compromised growth (Meaden *et al.*, 1990).

Kre5p shows some amino acid similarity with mammalian, insect, and *Schizosaccharomyces pombe* uridine diphosphate (UDP)–glucose:glycoprotein glucosyltransferase (GT) (Parker *et al.*, 1995; Fernandez *et al.*, 1996). Both proteins are approximately the same size and contain the ER-retrieval signal *HDEL* for soluble proteins at their C-terminal ends. GT transfers a single glucose unit to glucose-free protein-linked high-mannose-type oligosaccharides specifically on incompletely folded proteins (Sousa *et al.*, 1992; Fernandez *et al.*, 1994). It has been proposed that this enzyme is a key element of the so-called quality control of glycoprotein folding (Helenius *et al.*, 1997 and references therein). Monoglucosylated protein-linked oligosaccharides, arising by partial deglucosylation of the oligosaccharide Glc3Man9GlcNAc2 precusor, were shown to be recognized by ER lectins as membrane-bound calnexin or its soluble homologue calreticulin. Upon further deglucosylation by GII, glycoproteins would be liberated from their lectin anchors. If the released glycoprotein is not yet properly folded, it is then reglucosylated by GT, resulting in binding to the lectins. This deglucosylation–reglucosylation cycle can continue until proper folding is achieved. This interaction with the lectins apparently not only prevents exit of misfolded glycoproteins from the ER but also facilitates correct folding by hindering aggregation.

S. cerevisiae is the only organism described so far to be devoid of GT activity, not only in vitro but also in vivo. It was shown that monoglucosylated protein-linked oligosaccharides are not formed in *alg5* or *alg6* mutant cells that do not synthesize glucosylated lipid-linked oligosaccharides and transfer nonglucosylated oligosaccharides to protein (Fernandez *et al.*, 1994). Similar results were found upon incubation of *alg5gls2* double mutants (*GLS2* codes for GII) (Jakob *et al.*, 1998). *S. cerevisiae* not only lacks GT but also calreticulin, and its calnexin-like protein presents significant structural variations compared with its mammalian and *S. pombe* counterparts (Parlati *et al.*, 1995). Nevertheless, recent evidence indicates that monoglucosylated oligosaccharides do indeed play a role in facilitating glycoprotein folding in *S. cerevisiae* (Jakob *et al.*, 1998).

Because of its homology with GT, it has been proposed that Kre5p may be a glucosyltransferase involved in the initiation of β-1,6-glucan synthesis (Shahinian *et al.*, 1998). This possibility requires that UDP–glucose be present in the ER lumen. The purpose of this study was to investigate whether this sugar nucleotide is effectively transported from the cytosol, where it is synthesized, to the ER lumen. The only UDP-glucose–dependent reaction known so far to occur in *S. cerevisiae* ER is the formation of dolichol-P-glucose, and it apparently occurs on the cytosolic side of the ER membrane (Snider *et al.*, 1980; Hanover and Lennarz, 1982; Spiro and Spiro, 1985; Trombeta *et al.*, 1991; Abeijón and Hirschberg, 1992). We demonstrate UDP–glucose transport in vitro using a preparation enriched in ER vesicles. We also show UDP–glucose transport into the ER in vivo by demonstrating functional expression of *S. pombe* GT, an ER-luminal enzyme that requires UDP–glucose for activity. We also demonstrate that GT expression does not correct the *S. cerevisiae kre5*$^-$ phenotype.

MATERIALS AND METHODS

Strains and Growth Conditions

S. cerevisiae strains were grown in YPD or SD medium supplemented with the required auxotrophies. Solid medium was made by adding 2% agar to liquid medium. Standard procedures were used for genetic crosses, sporulation of diploids, and dissection of tetrads. Strains used for this study are isogenic with YPH274 (Sikorski and Hieter, 1989) except for PRY103 (Runge *et al.*, 1984). Strains HH2 y HH3 are haploids derived by sporulation of YPH274 (Table 1). Yeast and bacterial transformations were done by electroporation as described previously (Abeijón *et al.*, 1996). *Escherichia coli* DH5α cells were used for plasmid propagation and were grown in LB medium, containing Ampicillin (100 μg/ml) when needed (Maniatis *et al.*, 1982).

Disruption of the ALG5 Locus

The OCY1 *alg5::HIS3/ALG5* heterozygous strain was constructed via one-step gene replacement (Rothstein, 1983). A wild-type diploid YPH274 was transformed with a 2.8-kilobase (kb) *Bam*HI–*Hpa*I linear fragment from *palg5::HIS3* (te Heesen *et al.*, 1994) to histidine prototrophy. Sporulation yielded OCY2 haploid *alg5::HIS3* cells. Histidine prototrophs were analyzed for correct homologous recombination by PCR analysis with nucleotides 500–523 (5′-GCACAAAGGACCATAGTCACTGTG) as sense primer and nucleotides 1725–1746 (5′-AGCAAATGCCCTTGAGCGAG) of the *ALG5* gene

Table 1. *S. cerevisiae* strains used in this study

Strain	Genotype	Source
PRY103	*MATa ade 2-101 ura 3-52 alg 6-1*	P.W. Robbins
YPH274	*MATa/α ura3-52/ura3-52 lys2-801/lys2-801 lys 2-801 ade2-101/ade2-101his3-Δ200/his3-Δ200/trp1-Δ1/trp1-Δ1 leu 2-Δ1/leu 2-Δ1*	P. Hieter
HH2	*MATa ura3-52 lys2-801 ade2-101 his 3-Δ200 trp1-Δ1 leu2-Δ1*	This study
HH3	*MATα ura 3-52 lys 2-801 ade 2-101 his 3-Δ200 trp 1-Δ1 leu 2-Δ1*	This study
OCY1	YPH274 Δ*alg5::HIS3/ALG5*	This study
OCY2	HH2 Δ*alg5::HIS3*	This study
OCY3	HH2 Δ*alg5::HIS3* Δ*gls2::URA3*	This study
OCY5	YPH274 Δ*kre5::HIS3/KRE5*	This study
OCY6	HH2 Δ*kre5::HIS3*	This study

(GenBank accession no. X77573) as antisense primer or a *HIS3*-specific primer and the same antisense primer.

Disruption of the GLS2 Locus

A PCR fragment that included nucleotides 226–270 (5′-CTCACTACGGCACTTGAGAGCTATAACTCAATGAACATGTTGCTT) and nucleotides 3184–3225 (5′-GTTATTTTTTGAGGGAAAAAAACGAAGTGATATCTTTACATC) of the *GLS2* gene (GenBank accession no. Z36098) at the 5′- and 3′-ends of the *URA3* gene was generated (Lorenz *et al.*, 1995). OCY2 haploid *alg5::HIS3* cells were transformed with the 1255-base pair PCR product by electroporation to obtain the *alg5::HIS3 gls2::URA3* double-mutant OCY3 strain. Uracil and histidine prototrophs were analyzed for *GLS2* gene disruption using two primers proximal to those used for disruption, nucleotides 208–225 (5′-CCCGGACCACGACATCATTTT) as sense primer and nucleotides 3243–3266 (5′-ACGGATATTTTTACGTTTACTTTG) as antisense primer or one of these and a *URA3*-specific primer. OCY3 cells were completely devoid of GII activity when assayed with [glucose-^{14}C]$Glc_1Man_9GlcNAc$ as substrate.

Disruption of the KRE5 Locus

The *KRE5/kre5::HIS3* heterozygous strain was constructed via one-step gene replacement (Rothstein, 1993). The wild-type diploid YPH274 was transformed to histidine prototrophy with a *Pvu*II linear fragment derived from p*kre5::HIS3* (Meaden *et al.*, 1990). Plasmid p*kre5::HIS3* contains a disruption of *KRE5* gene in which a 3.0-kb *Eco*RI–*Bam*HI fragment had been replaced by a 1.8-kb fragment containing the *HIS3* gene. Diploid cells were sporulated, and *kre5::HIS3* haploid cells were obtained after tetrad dissection. A 2:2 segregation in colony size with two wild-type (*His*$^-$) and two microcolonies (*His*$^+$) was observed. Histidine prototrophs were analyzed for correct homologous recombination by PCR analysis using nucleotides 448–469 (5′-TAGGTACGAAAAGCCCGACGAC) as sense primer and either nucleotides 3776- 3800 (5′-CCCAGTATCCTTCTTTCCAATAACC) or nucleotides 1487–1510 (5′-TGCCTCCTTCACCTCACTATCTTC) as antisense primers. Positions correspond to GenBank M33556 sequence. Disruption of the *KRE5* locus was further confirmed by the total resistance to K1 killer toxin of the histidine prototrophs.

Expression of S. pombe GT in S. cerevisiae

Mutation of PDEL to HDEL Retrieval Signal. The entire *S. pombe*-encoding gene (*gpt1*$^+$) was cloned in three pieces. The 1338-nucleotide 5′-terminus fragment was obtained by digestion with *Bam*HI and *Nde*I of the 1475-nucleotide PCR product generated using the entire *gpt1*$^+$ cDNA in pBluescript (Fernández *et al.*, 1996) as template and primers 5′-GCT*GGATCC*ATGAGATGGGGCTTTTGGTTT (sense) and 5′-CATACCATACCAGTCTGAACGGG (antisense). The sense primer corresponded to nucleotides 751–771 of *gpt1*$^+$ (GenBank U38417) and in it a *Bam*HI (bases in italics) was created immediately before the ATG codon to allow cloning. The antisense primer corresponded to bases 2192–2216. The central fragment was obtained as a 2500-bp *Nde*I restriction fragment that comprised nucleotides 2089–4588 of *gpt1*$^+$. The 506-bp 3′-terminus fragment was obtained by digestion with *Nde*I and *Xho*I of the 665-nucleotide PCR product generated with primers 5′-AGCAACGTGAGATATGGGGATAC (sense, corresponding to nucleotides 4445–4467 of *gpt1*$^+$) and 5′-GCG*CTCGAG*TATGTTTCAAAGTTCGTC*ATG*AGATGAGTTGTTATC (antisense, corresponding to nucleotides 5065–5094 of *gpt1*$^+$). In the last primer both a *Xho*I site (first 6 bases in italics) and a mismatch (GGC to GTA, last three bases in italics) were created. The mismatch converted a P into an H in the ER-retrieval signal, and the *Xho*I site allowed cloning. The three fragments were ligated to a *Bam*HI and *Xho*I p426GPD-digested plasmid (Mumberg *et al.*, 1995) in a four-partner ligation to produce p426GPD-*gpt1*$^+$. The 4344-nucleotide fragment containing the complete *gpt1*$^+$ sequence with the mutated ER-retrieval signal was obtained by digestion of p426GPD-*gpt1*$^+$ with *Bam*HI and *Xho*I and introduced into p416GPD and p425GPD (Mumberg *et al.*, 1995) to yield p416GPD-*gpt1*$^+$ and p425GPD-*gpt1*$^+$. The p416GPD and p426GPD are low- and high-copy number plasmids, respectively, and both carry the *URA3* marker gene. On the other hand, p425GPD is a high-copy number plasmid that carries the *LEU2* marker gene. In the three plasmids, GT expression was under the glyceraldehyde 3-P dehydrogenase promoter.

Plasmid Construction: pKRE5

A 6.6-kb HindIII–*Sal*I fragment containing the *KRE5* gene with its own promoter was obtained from the pBluescript-*KRE5* (kindly provided by H. Bussey). It was cloned into the *Hin*dIII–*Sal*I sites at the multiple cloning site of vector YEp352 (Hill *et al.*, 1986).

RT PCR

RNA was extracted from cells in exponential phase at an OD_{600} of 0.4–0.6 as described previously (Collart and Oliviero, 1993). The primer used for reverse transcription corresponded to the antisense one used for synthesis of the 665-bp 3′-terminal fragment of gene gpt1$^+$. The same primer and the sense one used for synthesis of the same fragment were used for the PCR reaction.

Materials

[^{3}H]acetic acid, sodium salt (100 Ci/mol), GDP-[3,4-^{3}H]Man (20 Ci/mmol), UDP–*N*-acetyl[6-^{3}H]-D-GlcNAc (30 Ci/mmol), and [^{14}C]glucose (250 Ci/mol) were from Dupont/New England Nuclear (Boston, MA). [1,2-^{3}H]2-deoxyglucose (45 Ci/mmol), UDP–[6-^{3}H]Gal (60 Ci/mmol), and UDP–[1-^{3}H]glucose (15 Ci/mmol) were from American Radiochemicals (St. Louis, MO). Jack bean α-mannosidase and endo-β-*N*-acetylglucosaminidose H (Endo H) were

from Sigma Chemical (St. Louis, MO). UDP–[^{14}C]Glc (250 Ci/mol) was prepared as described by Wright and Robbins (1965).

Substrates and Standards

[Glucose-^{14}C]$Glc_1Man_9GlcNAc$, [glucose-^{14}C]$Glc_1Man_8GlcNAc$, [glucose-^{14}C]$Glc_1Man_7GlcNAc$, glucose-^{14}C]$Glc_1Man_4GlcNAc$, [glucose-^{14}C]$Glc_1Man_5GlcNAc$, [^{14}C]ManGlcNAc, [^{14}C]$Man_9GlcNAc$, [^{14}C]$Man_8GlcNAc$, and [^{14}C]$Man_7GlcNAc$ were prepared as described previously (Fernández *et al.*, 1994).

Preparation and Characterization of P_2 Fraction

The P_2 vesicle fraction used in sugar-nucleotide-translocation experiments was prepared as previously described with minor modifications (Abeijón *et al.*, 1989). Briefly, wild-type YPH274 yeast cells were grown in YPD medium to an OD_{600} of 3, centrifuged, and converted to spheroplasts using 10 mg of Zymolyase 100T (ICN, Richmond, CA) per 20 g of cells. Cells were broken by resuspension in buffer A (10 mM triethylamine-acetate, pH 7.2/0.8 M sorbitol/1 mM EDTA) (1 ml/g of cells) and by drawing the suspension rapidly several times into a small-bore serological pipette. Pepstatin (6 μg/ml) and PMSF (1 mM) were used as protease inhibitors. Cell breakage was not complete with these heavy suspensions, but vesicle integrity was very well preserved. The lysate was centrifuged at 1500 $\times$ *g* for 20 min to give a first pellet P_1 and a supernatant. This supernatant was centrifuged at 100,000 $\times$ *g* for 1 h to obtain P_2 vesicle fraction. This P_2 pellet was gently resuspended in buffer A at 10–15 mg protein/ml, aliquoted, and immediately frozen in liquid nitrogen. Each aliquot was thawed only once to preserve vesicle integrity. The specific activity of NADPH-cytochrome C reductase, an ER marker enzyme, was 0.14 nmol/mg of protein/min, representing a 3.1-fold enrichment over homogenate (63% recovery of total homogenate activity). The assay was done as described by Kubota (1977). The luminal Golgi marker guanosine diphosphatase (GDPase) (Abeijón *et al.*, 1989) was 2.6-fold enriched in the P_2 fraction (specific activity: 1.4 mmol of GDP hydrolyzed/mg of protein/min; 48% of total homogenate activity). Vesicle integrity of the P_2 fraction was at least 90% as determined my measuring latency of GDPase activity.

Nucleotide-Sugar-Translocation Assay

The theoretical basis and calculations for the translocation assay into vesicles of nucleotide derivatives have been described previously in detail (Pérez and Hirschberg, 1987). Briefly, it consists of 1) determining the total radioactive solutes associated with a vesicle pellet (St) after incubation of vesicles with radioactive substrates and centrifugation and 2) substracting from this value the total radioactive solutes outside the vesicles in the pellet (So, see below). This yields the total radioactive solutes within vesicles (Si). Incubations were done in 1 ml of buffer B (30 mM triethanolamine-acetate [pH 7.2]/0.3 M sucrose/5 mM $MgCl_2$/5 mM $MnCl_2$). The specific activity of the different radioactive substrates was adjusted to 3000 cpm/pmol. Incubations were initiated by the addition of 0.8–1.5 mg of P_2 vesicle protein per assay, derived from wild-type (YPH274) cells. At the end of the incubations, 3 ml of ice-cold 0.5 M sucrose were added, and the reaction mixtures were immediately centrifuged for 25 min at 100,000 $\times$ *g*.

Calculations Used to Determine Nucleotide Sugar Translocation

Sm = concentration of solute in the incubation medium [μM]= counts per min/ml of solute in the supernatant/specific activity of solute (expressed as counts per min/nmol). St= total solute in the pellet (expressed in picomoles/mg of protein) = total soluble radioactivity associated with the pellet (expressed as counts per min/mg of protein)/specific activity of solute (expressed as counts per min/pmol). So = solutes outside the vesicles in the pellet (picomoles/mg of protein) = Vo (microliters/mg of protein) $\times$ Sm (picomoles/μl). Vt, the total volume in the pellet (both inside and outside the vesicles) available to solutes when 1 mg of vesicle protein was used, was calculated using the total counts per min/mg protein obtained after incubating with 2-deoxy-D-[^{3}H]Glc, a penetrant solute that is evenly distributed both inside and outside the vesicles. A Vt of 2.65 μl/mg protein was measured for the P_2 vesicle fraction used in these studies. Vo, the volume external to the vesicles in the pellet, was calculated in the same manner using data obtained from the incubation with [^{3}H]acetate, a solute that cannot enter the vesicles and is, therefore, distributed only in the pellet volume external to vesicles. Vo = (counts per min/mg protein in the pellet)/(counts per min/ml in the supernatant). Vi, the internal volume of the vesicles in the pellet, equals Vt $-$ Vo. Vo was calculated to be 1.45 μl/mg protein and Vi = 1.2 μl/mg protein. [Si] is the concentration of solute inside the vesicles, which equals Si/Vi.

In Vivo Labeling of S. cerevisiae Cells

Labeling of *S. cervisiae* cells with [^{14}C]glucose was performed as described earlier but 150 μCi of [^{14}C]glucose were used (Fernández *et al.*, 1994). Incubation with the label lasted for 15 min. Where indicated, 5 mM 1-deoxynojirimycin (DNJ) was added during the 15-min preincubation and 15-min incubation. Isolation of Endo H-sensitive oligosaccharides was performed as described previously (Fernández *et al.*, 1994).

Carbohydrate Methods

Strong acid hydrolysis and treatment of oligosaccharides with Jack bean α-mannosidase were as described previously (Fernández *et al.*, 1994). Whatman 1 papers were used for chromatographies. Solvents employed were: A, 1-propanol/nitromethane/water (5:2:4); B, 1-butanol/pyridine/water (4:3:4); and C, 1-butanol/pyridine/water (10:3:3). GII activity was assayed as described previously using [glucose-^{14}C]$Glc_1Man_{7-9}GlcNAc$ as substrate (Ugalde *et al.*, 1979). GT was assayed using *S. cerevisiae* cell microsomes as enzyme source and 8 M urea-denatured thyroglobulin as acceptor as previously described (Fernández *et al.*, 1994).

RESULTS

Transport of UDP–Glucose into S. cerevisiae Microsomal Vesicles

To determine whether UDP–glucose is transported into the lumen of *S. cerevisiae* ER-derived vesicles, a vesicle-enriched population, which was sealed and of the same membrane orientation as in vivo, was incubated with UDP–[^{3}H]glucose. The suspension was centrifuged, and the radioactive solutes that had accumulated in the vesicle pellet were measured (Table 2). Total radioactive solutes in the pellet (St) were 55.4 pmol/mg protein after a 3-min incubation. Only a small amount of the total solutes in the pellet was outside, in between the vesicles in the pellet (So), and ~95% of total radioactive solutes were within the vesicles in the pellet (Si). Detailed calculations are described in MATERIALS AND METHODS. Table 2 also shows that incubation of vesicles with UDP–[^{3}H]glucose resulted in a 22-fold accumulation of radiolabeled solutes within vesicles as compared with the concentration of radiolabeled solutes remaining in the incubation medium. Transport of UDP–glucose

Table 2. Translocation of UDP-glucose into an ER- and Golgi-enriched vesicle fraction[a]

Substrate	Temp (°C)	[Sm] (μM)	Solute (pmol/mg protein) St	So	Si	[Si] (μM)	[Si]/[Sm]
UDP-Glc	30	2	55.4	2.9	52.5	43.7	21.9
UDP-Glc	0	2	6.1	2.9	3.2	2.7	1.35
UDP-Glc[b]	30	2	2.3	2.9	0	0	0
UDP-GlcNAc	30	2	3.2	2.9	0.3	0.2	0.1
UDP-Gal	30	2	12.4	2.9	9.5	7.9	3.9
GDP-Man	30	2	39.7	2.9	36.8	30.7	15.3
Glucose	30	4	10.7	5.8	4.9	4.1	1

[a] Radioactive substrates (3000 cpm/pmol) were incubated for 3 min at the indicated temperatures with P_2 vesicle fraction (1.5 mg of protein) in 1 ml of buffer B. Translocation was determined by a centrifugation assay as described in detail (34). Sm and [Si] are, respectively, solutes in the medium and within vesicles. St, So, and Si are respectively, total solute and solute outside and inside vesicles. Calculations are described in Materials and Methods. Results are average of triplicate determinations.

[b] Triton X-100, 0.05%, was added to the assay.

was temperature dependent, and the amount of solutes detected inside the vesicles at 0°C was only 6% of that at 30°C (Table 2). The transport signal was dependent on the integrity of the vesicles, because when a very small amount of detergent (0.05% Triton X-100) was added, no solutes were found inside the vesicles (Table 2). This mild detergent treatment only permeabilizes the vesicles and does not extract membrane proteins; upon centrifugation, the membrane enzyme recovery of GDPase in the pellet was 87–95% of that in untreated samples (our unpublished data).

The accumulation of UDP–[^{3}H]glucose–derived solutes within the lumen of the vesicles was dependent on protein in a linear manner, between 0.8 and 1.5 mg and on time, up to 6 min (our unpublished data). The transport reaction was saturable with an apparent K_m of 4.6 μM and a V_{max} of 200 pmol/mg protein/3 min (Figure 1). After the 3-min incubation, 90–95% of the radioactivity in the incubation medium was UDP–glucose. Transport of UDP–glucose into the lumen of the vesicles was specific because another uridine nucleotide sugar such as UDP–*N*-acetyl-glucosamine was not transported (Table 2). Incubations with UDP–[^{3}H]galactose resulted in 9.5 pmol/mg protein of radioactive solutes accumulating inside the vesicles (Si) and a modest fourfold concentration over the incubation medium (Table 2). The small signal detected for UDP–galactose is in agreement with a previous report for the existence of an UDP–galactose transporter in *S. cerevisiae* (Roy *et al.*, 1998), although no endogenous acceptors for galactose have been described in this organism to date. No accumulation of radioactive solutes was detected inside the vesicles when they were incubated with [^{3}H]glucose (Table 2). GDP–mannose, a nucleotide sugar that is transported mainly into the lumen of the Golgi apparatus in *S. cerevisiae* (Abeijón *et al.*, 1989), was transported by this mixed vesicle fraction, resulting in a 15-fold accumulation of solutes within the vesicles over that in the incubation medium (Table 2). The following experiments were designed to demonstrate that UDP–glucose was transported into the ER in vivo.

Expression of S. pombe GT in S. cerevisiae

Cell-free Assay. Before determining in vivo expression of S. pombe GT as a means to detecting transport of

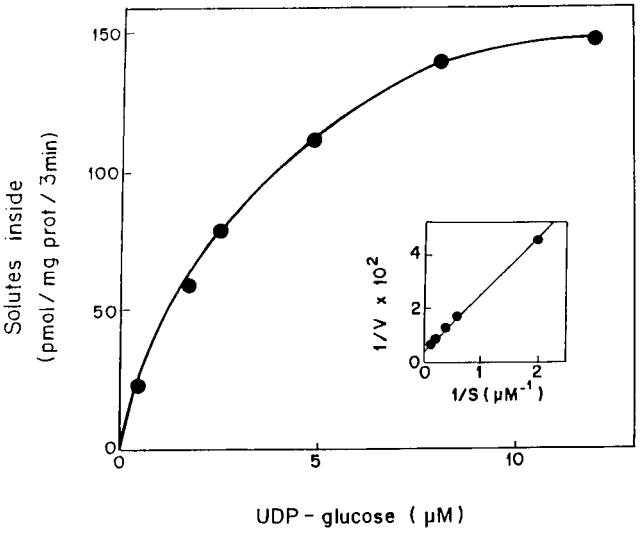

Figure 1. Rate of solutes accumulation within vesicles versus UDP–glucose concentration in the incubation medium. A P_2 vesicle fraction (see MATERIALS AND METHODS) (1.2 mg of protein) was incubated at 30°C for 3 min with 1 μCi of UDP–[^{3}H]glucose plus different amounts of the unlabeled nucleotide sugar to give the desired final concentrations. Translocation results shown are the mean of two separate determinations. Inset, Double-reciprocal plot according to Lineweaver and Burk.

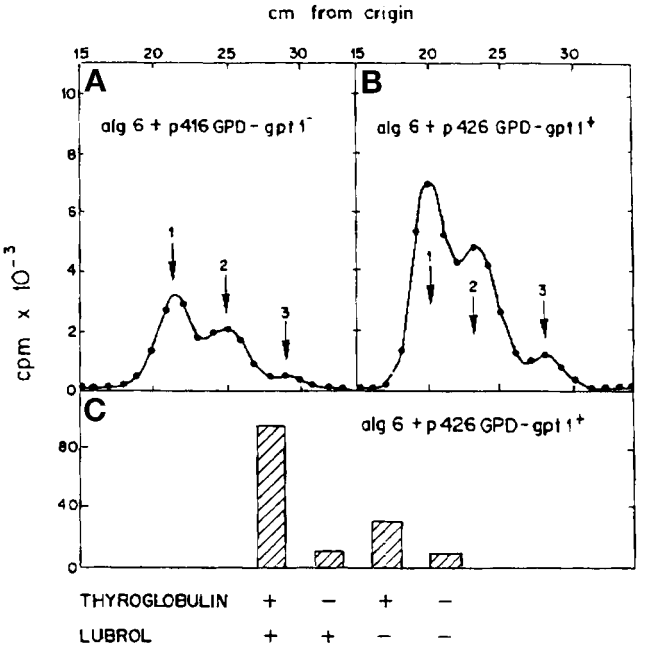

Figure 2. Cell-free assays of *S. pombe* GT expressed in *S. cerevisiae*. Microsomes from *S. cerevisiae alg6* (PRY103) mutant cells transformed with p416GPD-*gpt1*$^+$ (A) or p426GPD-*gpt1*$^+$ (B) were incubated with UDP–[^{14}C]glucose in the presence of 8 M urea-denatured thyroglobulin. Resulting Endo H-sensitive oligosaccharides were run on paper chromatography with solvent A. In panel C, thyroglobulin or detergent was omitted where indicated. Standards: 1, $Glc_1Man_9GlcNAc$; 2, $Glc_1Man_8GlcNAc$; and 3, $Glc_1Man_7GlcNAc$.

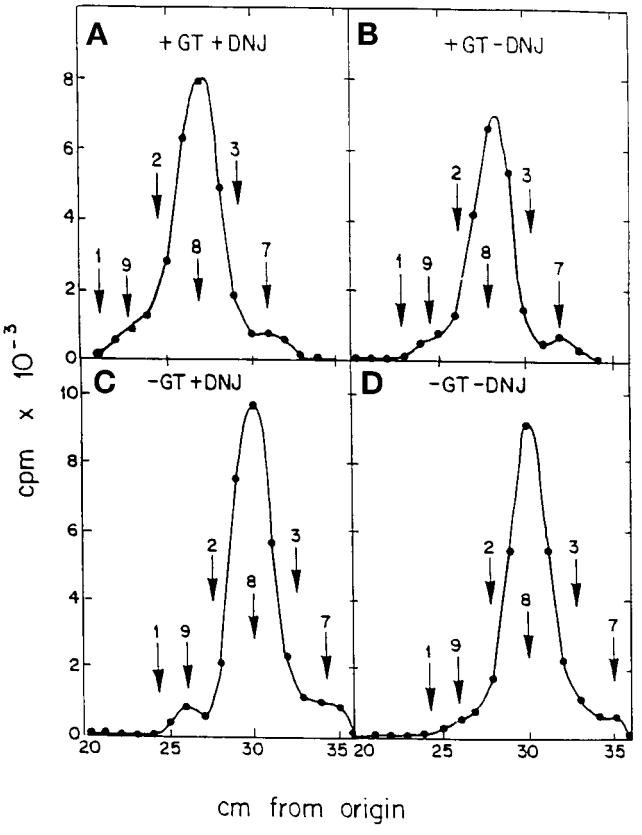

Figure 3. Intact-cell assay of *S. pombe* GT expressed in *S. cerevisiae*. *S. cerevisiae alg6* mutant (PRY103) cells transformed with p426GPD-*gpt1*$^+$ (A and B) or with p426GPD (C and D) were incubated with [^{14}C]glucose for 15 min in the presence (A and C) or absence (B and D) of DNJ. Resulting Endo H-sensitive oligosaccharides were run on paper chromatography with solvent A. Standards: 1, $Glc_1Man_9GlcNAc$; 2, $Glc_1Man_8GlcNAc$; 3, $Glc_1Man_7GlcNAc$; 9, $Man_9GlcNAc$; 8, $Man_8GlcNAc$; and 7, $Man_7GlcNAc$.

UDP–glucose into the ER lumen, we performed experiments to determine its in vitro expression. The C-terminal end of *S. pombe* GT was mutated from PDEL to the *S. cerevisiae* retrieval signal HDEL, and the GT-encoding gene, *gpt1*$^+$, was then inserted in low- and high-copy number expression vectors (p416GPD and p426GPD, respectively) under the glyceraldehyde 3-P dehydrogenase promoter. *S. cerevisiae alg6* cells (PRY103) were then transformed with the expression vectors with or without *S. pombe gpt1*$^+$ gene. The *alg6* mutant was chosen to ensure that any glycoprotein glucosylation observed would be independent from intermediates involving dolichol derivatives. This mutant is defective in the dolichol-P-glucose-dependent glucosyltransferase that transfers the first glucose unit to $Man_9GlcNAc_2$-P-P-dolichol. The microsomal fractions from cells transformed with the above-mentioned plasmids were incubated with UDP–[^{14}C]glucose in the presence of 8 M urea-denatured thyroglobulin. Total proteins were degraded with an nonspecific protease, and resulting glycopeptides were digested with Endo H. Only microsomes derived from cells transformed with the GT-encoding plasmids yielded the expected products, $Glc_1Man_9GlcNAc$, $Glc_1Man_8GlcNAc$, and $Glc_1Man_7GlcNAc$ (Figure 2, A and B). No labeled glycopeptides, where detected when the *alg6* cells were transformed with the expression vectors without the *S. pombe gpt1*$^+$ gene, confirmed the absence of endogenous GT activity in *S. cerevisiae*. The GT-specific activities found with both the low- and high-copy number plasmids were higher than those present in wild-type *S. pombe* microsomes (Fernández *et al.*, 1994). The enzyme was present in vesicles as omission of detergents from the incubation mixtures significantly decreased incorporation into denatured thyroglobulin (Figure 2C).

In Vivo Assay. We wanted to determine whether glucosylation of lumenal N-linked oligosaccharides occurred in vivo, as this would indicate that UDP–glucose was transported into the ER where GT resides. Thus, alg6 cells (PRY103) transformed with plasmids p426GPD or p426GPD-*gpt1*$^+$ were incubated with [^{14}C]glucose for 15 min in the presence or absence of GII inhibitor DNJ. *N*-linked oligosaccharides were liberated as described for experiments depicted in Fig-

ure 2, A and B. Similar patterns were obtained in all cases (Figure 3, A–D). They showed the presence of oligosaccharides migrating as $Man_9GlcNAc$, $Man_8GlcNAc$, and $Man_7GlcNAc$ standards. No peaks or shoulders migrating as their monoglucosylated derivatives were observed, indicating that only minimal amounts of those structures might be present. It has been described that demannosylation of the oligosaccharide transferred to proteins stops at the $Man_8GlcNAc$ stage in *S. cerevisiae* (Byrd *et al.*, 1982). Nevertheless, we have always observed small amounts of the compound having 7 mannose units. Upon degradation of compounds depicted in Figure 3, A–D, with Jack bean α-mannosidase, all samples yielded mannose and the disaccharide ManGlcNAc (Figure 4, A–C; the pattern obtained from samples of cells transformed by p426GPD incubated without DNJ was similar to that shown in Figure 4C). The presence of ManGlcNAc among the degradation products is indicative of the presence of nonglucosylated oligosaccharides.

Samples from cells transformed with the GT-encoding plasmid produced, in addition to the oligosaccharides shown above, an α-mannosidase–resistant compound that migrated as a $Glc_1Man_4GlcNAc$ standard (Figure 4, A and B). The amount of the α-mannosidase-resistant core was much higher in the sample from cells incubated with DNJ. Strong acid hydrolysis of the core produced labeled glucose and mannose units (Figure 4D). The relative proportion of glucosylated and nonglucosylated oligosaccharides in Figure 3A was calculated as previously described (Gañán *et al.*, 1991). Briefly, the label in mannose in ManGlcNAc divided by the sum of the label in mannose in the same compound plus that in mannose in $Glc_1Man_4GlcNAc$ divided by 4 gave the relative proportion of nonglucosylated compounds. No labeled glucosamine appeared in the pattern shown in Figure 4D, thus indicating that those residues were not significantly labeled during the 15-min incubation with [^{14}C]glucose. This facilitated quantitation of mannose label in ManGlcNAc. The relative proportion of glucosylated compounds was found to be ~7%. The presence of glucosylated oligosaccharides in the ER lumen, therefore, indicated that UDP–glucose was indeed transported into this compartment in vivo.

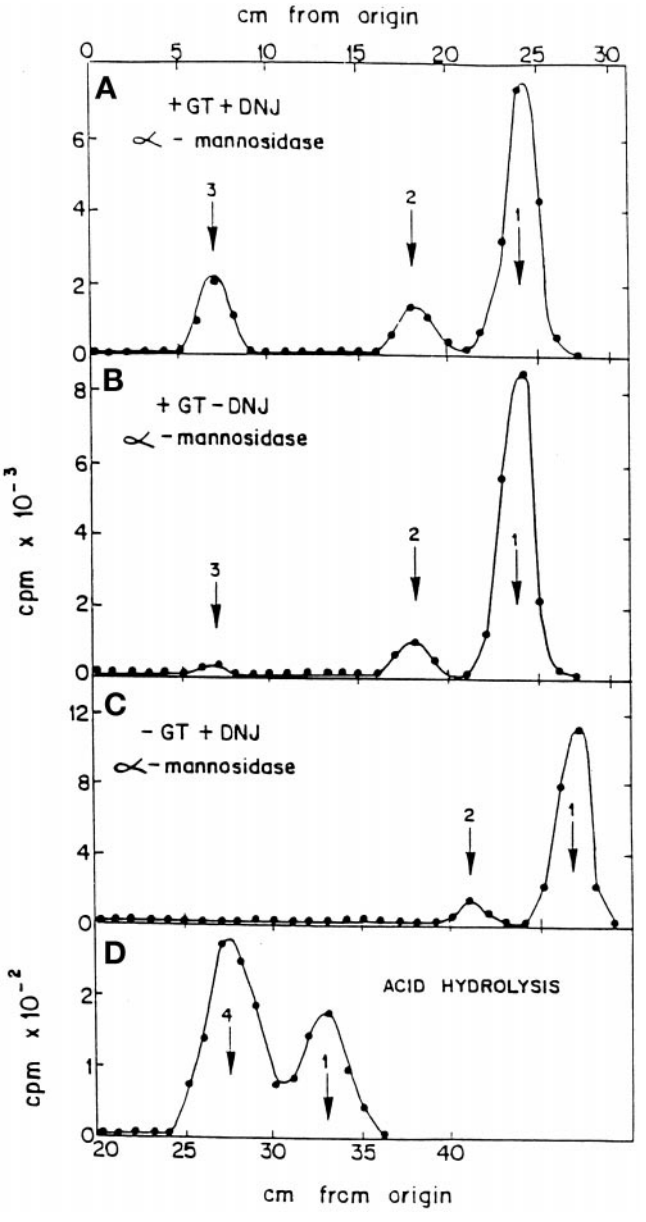

Figure 4. Structural characterization of oligosaccharides. Compounds shown in Figure 3, A–C, were digested with α-mannosidase and run on paper chromatography with solvent B (A–C, respectively). Compound migrating as standard 3 in panel A was submitted to strong acid hydrolysis and run on paper chromatography with solvent C. Standards: 1, mannose; 2, ManGlcNAc; 3, $Glc_1Man_4GlcNAc$; and 4, glucose.

Oligosaccharide Glucosylation in GII Minus Cells

The unexpected low proportion of glucosylated oligosaccharides found above could have been the result of only a partial inhibition of GII by DNJ. Therefore, both *alg5* single- and *gls2 alg5* double-mutant cells transformed with p425GPD-*gpt1*$^+$ were incubated with [^{14}C]glucose for 15 min in the presence or absence of DNJ. The *gls2 alg5* double mutant was also transformed with p425GPD as a control. In *alg5* cells, as in *alg6*, $Man_9GlcNAc_2$ is transferred to proteins as the *alg5* cells are unable to synthesize dolichol-P-glucose (Runge *et al.*, 1984). The above double-mutant cells lack GII in addition to dolichol-P-glucose synthetase. The patterns of Endo H-released compounds were similar, if not identical, to those shown in Figure 3, A–D. Substances migrating between the middle of $Man_9GlcNAc$ and $Man_8GlcNAc$ standards (i.e., in the position expected for the principal glucosylated com-

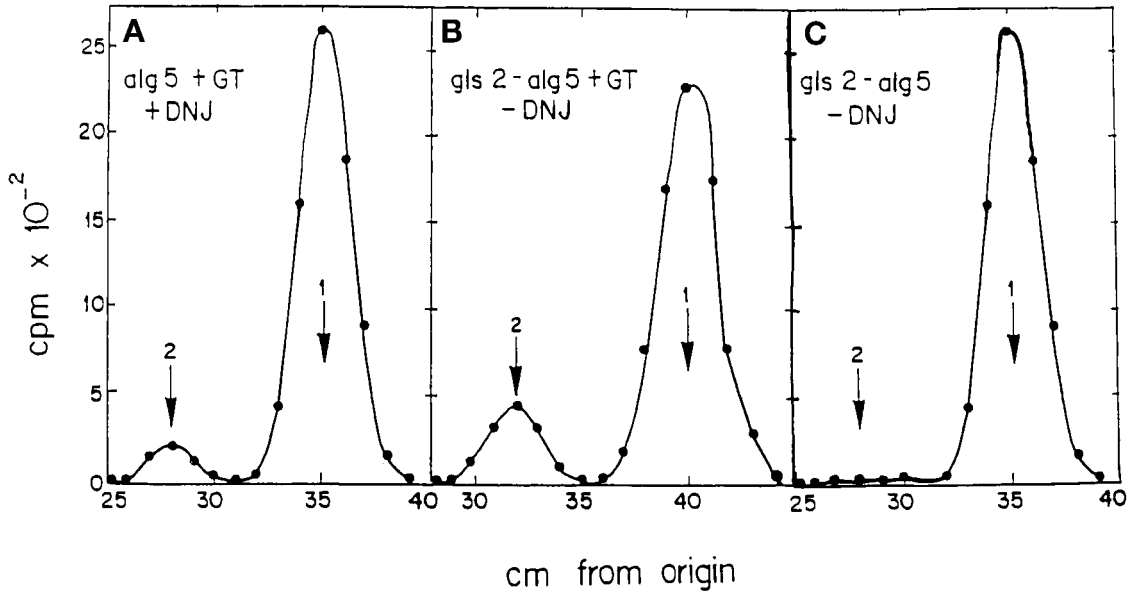

Figure 5. GT expression in GII minus cells. Alg5 (OCY2) or *gls2-alg5* (OCY3) cells transformed with p425GPD-*gpt1*$^+$ or *p425 GPD* were incubated for 15 min with [^{14}C]glucose in the presence or absence of 5 mM DNJ. Endo H-liberated compounds were run on paper chromatography with solvent A. Material migrating between mid-$Man_9GlcNAc$ and $Man_8GlcNAc$ was submitted to strong acid hydrolysis and run on paper chromatography with solvent C. (A) *alg5* cells with p425GPD-*gpt 1*$^+$ incubated with DNJ and (B) *gls2-alg5* with p425GPD-*gpt*$^+$ cells incubated without DNJ. (C) *alg5-gls2* cells with p425GPD incubated with DNJ. Standards: 1, mannose; and 2, glucose.

pound, $Glc_1Man_8GlcNAc$) were subjected to strong acid hydrolysis. As expected, the single mutant yielded labeled glucose residues only when incubated in the presence of DNJ (Figure 5A). The double mutant produced similar amounts of labeled glucose when incubated in the presence or absence of DNJ, thus confirming the lack of GII activity in the strain. The pattern yielded by the double mutant in the absence of DNJ is depicted in Figure 5B. When the *gls2 alg5* double mutant was transformed with p425 GPD without insert, only mannose was detected (Figure 5C). The proportion of label in glucose over that in mannose units was 6 and 13% in Figure 5, A (*alg5* plus DNJ) and B (*gls2 alg5* minus DNJ), respectively, thus indicating that the percentage of glucosylated oligosaccharides observed in experiments shown in Figures 3 and 4 (7%) could, at the most, be ~15% if complete inhibition of GII by DNJ had been achieved.

The above described results show that detection of glucosylated oligosaccharides was dependent on 1) expression of *S. pombe* GT and 2) inhibition of GII activity either by the addition of DNJ or by disruption of the enzyme-encoding gene. These results show, therefore, that UDP–glucose transport into the ER of *S. cerevisiae* occurred, as GII is precisely located in the lumen of this organelle. Moreover, the results presented indicate that a low percentage of N-linked oligosaccharides was glucosylated under nonstressed conditions.

GT Expression on the kre5$^-$ *Phenotype*

Kre5p displays a relatively high homology to *S. pombe* GT: it resides in the same subcellular compartment and has the same size. To test whether the *S. pombe* GT could correct the *kre5*$^-$ phenotype, *KRE5* was disrupted in a strain whose genetic background allows growth of mutant cells, although at a highly reduced rate. The growth rate of *kre5* mutant is identical with that of the same mutant transformed with YEp352 shown in Figure 6; mutant cells were completely resistant to K1 killer toxin as previously shown (Meaden *et al.*, 1990). As expected, transformation of *kre5* mutant cells with an expression vector carrying wild-type *KRE5* gene (pKRE5) corrected the slow-growth-rate phenotype (Figure 6) and recovered the sensitivity to K1 killer toxin to the level of wild type (our unpublished data). Neither recovery of the normal growth rate nor sensitivity to the toxin was observed upon transformation of *kre5* mutant cells with p425GPD-*gpt1*$^+$ (Figure 9). RT-PCR analysis of RNA extracted from *kre5* cells transformed with p425GPD-*gpt1*$^+$ indicated that *gpt1*$^+$ was effectively transcribed (Figure 6, inset). Taken together, these results indicate that *S. pombe* GT and Kre5p have different functions in spite of their similarities.

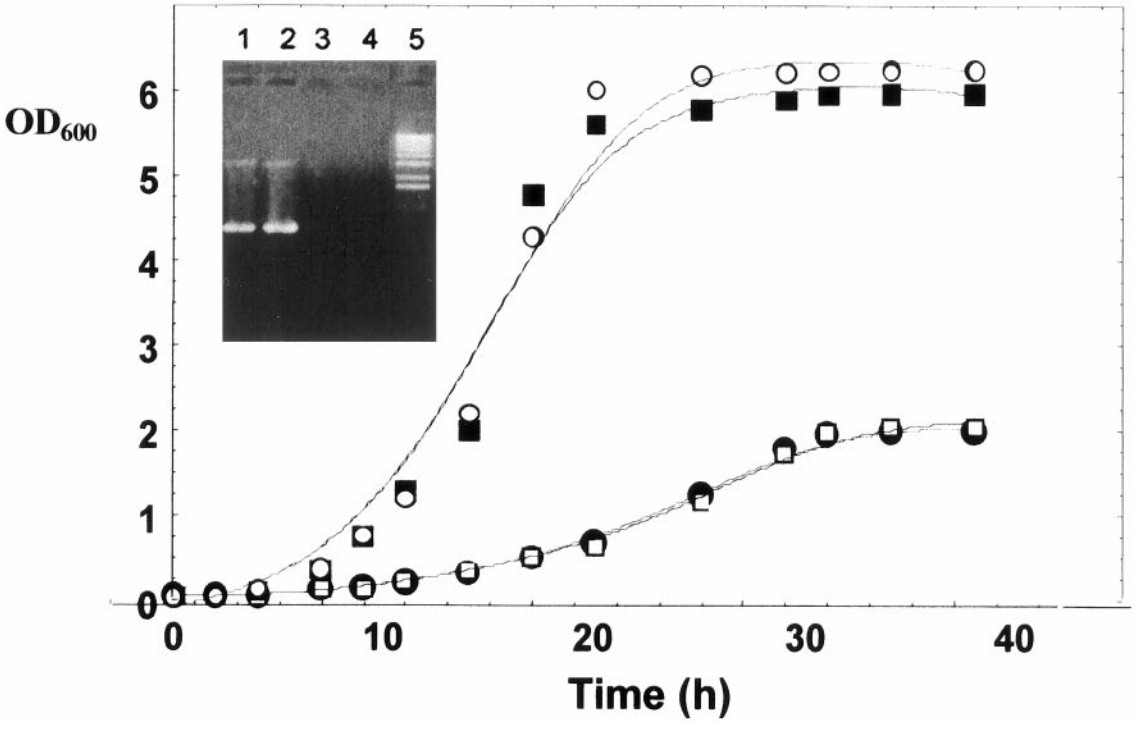

Figure 6. Effect of GT expression on the Kre5$^-$ phenotype. Growth of HH3 cells (open circles), of a *kre5::HIS3* mutant derived from it (OCY6) transformed with YEp352 (full circles), with pKRE5 (full squares) or with p425GPD-*gpt1*$^+$ (open squares). Inset, RT-PCR analysis of RNA synthesized by two clones of *kre5* mutant cells transformed with p425GPD-*gpt1*$^+$. Primers used corresponded to the end fragments of the 665-bp 3′-terminus of *gpt1*$^+$ (see MATERIALS AND METHODS). Lanes 1 and 2, complete system; lanes 3 and 4, omission of reverse transcriptase; lane 5, molecular weights markers.

DISCUSSION

This study demonstrates that UDP–glucose is transported into the *S. cerevisiae* ER in vivo and in vitro. Transport was saturable and temperature dependent with kinetic properties similar to those measured for the rat liver ER UDP–glucose transporter (Pérez and Hirschberg, 1986). Mammalian and yeast transporter proteins concentrated the substrate ~20 fold over the concentration present in the incubation medium, both exhibited apparent *K*m for UDP–glucose between 4 and 4.6 μM and comparable maximum velocities. No signal was detected for transport of UDP–GlcNac in *S. cerevisiae.* This was expected because *S. cerevisiae* lacks the terminal *N*-acetylglucosamine residues present in *Kluyveromyces lactis* mannan for which the UDP–GlcNac transporter provides the substrate (Abeijón *et al.*, 1996).

Because no UDP-glucose–dependent reaction has been described to occur in the lumen of the ER in *S.cerevisiae,* we used the heterologous expression of *S. pombe* GT as a means to detect in vivo transport of UDP–glucose into the ER. The GT transfers glucose from UDP–glucose to misfolded, but not native, glycoproteins and provides a recognition tag for chaperone systems to assist newly synthesized proteins acquiring their final native conformation (Helenius *et al.*, 1997). UDP–glucose was effectively transported to the ER lumen in vivo as indicated by the detection of monoglucosylated, high-mannose type protein-linked oligosaccharides in *S. cerevisiae alg5* or *alg6* mutants transformed with *S. pombe* GT expression vectors. Moreover, this detection was dependent on GII inhibition or on disruption of the gene encoding GII, the ER-located enzyme that is responsible for removal of the glucose unit transferred by GT. Because $Man_9GlcNAc_2$ is transferred from dolichol-P-P derivatives to nascent polypeptide chains in *alg5* and *alg6* mutants, glucose residues detected had to be necessarily added by GT.

The low proportion of glucosylated oligosaccharides detected was surprising in view of 1) the ample supply of UDP–glucose available in the lumen of the ER, and 2) the fact that the in vitro activity detected for the heterologously expressed GT was even higher then that measured in *S. pombe* microsomes directly. The proportion of glucosylated oligosaccharides did not increase when we induced glycoprotein misfolding by DTT or heat shock; suggesting that a misfolded pro-

tein conformation is not a sufficient condition for in vivo GT-dependent glycosylation of oligosaccharides, contrary to what occurs in vitro.

The role of UDP–glucose in *S. cerevisiae* ER lumen is presently unknown because this yeast lacks GT (Fernández *et al*., 1994; Jakob *et al*., 1998). It was reported many years ago that incubation of a crude bakers yeast microsomal fraction with UDP–[^{14}]glucose led to the synthesis of both charged (5% of total) and uncharged (95% of total) lipid derivatives. The first one was found to be dolichol-P-glucose, whereas the latter was tentatively identified as sterylglucoside (Parodi, 1976, 1977). Synthesis of this lipid could, therefore, require UDP–glucose in the ER lumen.

When the transport of UDP–glucose into the lumen of the mammalian ER was demonstrated (Pérez and Hirschberg, 1986), the physiological role of a lumenal pool of UDP-glucose was unknown. It was speculated that the formation of dolichol-P-glucose in the ER could be a lumenal or cytosolic reaction (Pérez and Hirschberg, 1986). Now there is consensus that this reaction occurs on the cytosolic side of the ER membrane (Snider *et al*., 1980; Hanover and Lennarz, 1982; Spiro and Spiro, 1985; Trombeta *et al*., 1991). Transient glucosylation of oligosaccharides by GT was discovered in 1983 (Parodi *et al*., 1983), and subsequently found to be widespread in nature (Trombeta *et al*., 1989). The physiological role of this transient reglucosylation reaction remained unknown for some time. It is now well established that the monoglucosylated oligosaccharides generated by GT in incompletely folded glycoproteins are recognized by chaperones that assist them in acquiring their mature conformation (reviewed by Helenius *et al*., 1997). This reaction explains the occurrence of a luminal pool of UDP–glucose in the ER.

There should be another reason for the existence of UDP–glucose transport into the ER of *S. cerevisiae* because it lacks GT (Fernández *et al*., 1994; Jakob *et al*., 1998). One possibility could be that synthesis of cell wall β-1,6- glucan is initiated in the ER lumen by Kre5p, as recently suggested (Shahinian *et al*., 1998). Results presented here show that although Kre5p displays a certain homology with *S. pombe* GT, the latter could not revert the *kre5*$^{-}$ phenotype, thereby demonstrating that both proteins have clearly different roles. This conclusion is in agreement with other recent findings by Shahinian *et al*., (1998), who constructed a strain that constitutively retains one glucose per N-chain in the same position in which GT would add it. This was achieved by a combination of *alg8* deletion, which prevents addition of the second glucose to the dolichol oligosaccharide, and a *glsII*Δ mutation, which prevents the removal of this glucose after the N-linked oligosaccharide has been transferred to protein. Because *kre5*Δ disruptions are lethal in a SEY6210 background, it was reasoned that if Kre5p were simply a GT, *kre5*Δ *alg8*Δ *gls2*Δ cells should be viable because the GT activity is no longer required in this strain that constitutively express glucose-containing oligosaccharides. The triple mutant was not viable despite the constitutive expression of monoglucosylated N-linked chains, showing that the essential function of Kre5p is unrelated to N-chain reglucosylation (Shahinian *et al*., 1998).

The role of Kre5p in the biosynthesis of β-1,6-glucan remains to be established; no enzymatic activity has yet been assigned to it. We have now demonstrated that an ample supply of UDP–glucose exists in the lumen of the ER of *S. cervisiae* where it could serve as substrate for putative β-1,6-glucan synthases. Only cloning of the gene(s) encoding the UDP–glucose transporter protein(s) and phenotypic analysis of null mutants will allow unequivocal determination of whether or not UDP–glucose transport is a prerequisite for the biosynthesis of cell wall β-1,6-glucan.

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