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# Isolation of the *ALG6* locus of *Saccharomyces cerevisiae* required for glucosylation in the N-linked glycosylation pathway

**Gilles Reiss, Stephan te Heesen, Janet Zimmerman[1], Phillips W.Robbins[1] and Markus Aebi[2]**

Mikrobiologisches Institut, Eidgenössische Technische Hochschule, ETH Zentrum, CH-8092 Zürich, Switzerland and [1]Center for Cancer Research, Massachusetts Institute of Technology, Cambridge, MA 02139, USA

[2]To whom correspondence should be addressed

**N-Linked protein glycosylation in most eukaryotic cells initiates with the transfer of the oligosaccharide $Glc_3Man_9GlcNAc_2$ from the lipid carrier dolichyl pyrophosphate to selected asparagine residues. In the yeast *Saccharomyces cerevisiae, alg* mutations which affect the assembly of the lipid-linked oligosaccharide at the membrane of the endoplasmic reticulum result in the accumulation of lipid-linked oligosaccharide intermediates and a hypoglycosylation of proteins. Exploiting the synthetic growth defect of *alg* mutations in combination with mutations affecting oligosaccharyl transferase activity (Stagljar *et al.*, 1994), we have isolated the *ALG6* locus. *alg6* mutants accumulate lipid-linked $Man_9GlcNAc_2$, suggesting that this locus encodes an endoplasmic glucosyltransferase. Alg6p has sequence similarity to Alg8p, a protein required for glucosylation of $Glc_1Man_9GlcNAc_2$.**

*Key words: Saccharomyces cerevisiae*/endoplasmic reticulum/glycosyltransferase/dolichol

## Introduction

N-Linked glycosylation of proteins initiates in the endoplasmic reticulum. In a highly conserved process, a core oligosaccharide is assembled at the ER membrane on the lipid-carrier dolichyl pyrophosphate (Kornfeld and Kornfeld, 1985). The core oligosaccharide $Glc_3Man_9GlcNAc_2$ is transferred to selected asparagine residues of nascent polypeptide chains by the oligosaccharyl transferase complex. The initial steps of the lipid-linked oligosaccharide assembly occur at the cytoplasmic side of the ER membrane, where nucleotide-activated sugars serve as substrates. Current models suggest that the lipid-linked intermediate $Man_5GlcNAc_2$ is translocated into the lumen of the ER, where an additional four mannose residues and three glucose residues are transferred from dolichylphosphomannose or dolichylphosphoglucose, respectively (Abeijon and Hirschberg, 1992). The subsequent addition of the three glucose residues, two α-1,3-linked, the terminal glucose α-1,2-linked, represents the final stage of the lipid-linked oligosaccharide assembly (Kornfeld and Kornfeld, 1985; Herscovics and Orlean, 1993). Fully glucosylated oligosaccharide is the preferred substrate for the oligosaccharyl transferase, but nonglucosylated oligosaccharides can be transferred to protein, albeit with a highly reduced efficiency (Turco *et al.*, 1977; Staneloni *et al.*, 1980; Trimble *et al.*, 1980; Murphy and Spiro, 1981; Sharma *et al.*, 1981). The nonglucosylated oligosaccharides are the natural substrates in some protozoan species (Parodi, 1993).

Three different glucosyltransferases are involved in the terminal glucosylation process in higher eukaryotes (Dsouzaschorey and Elbein, 1993) and different mutations affecting the addition of glucose residues have been identified in the yeast *Saccharomyces cerevisiae*. *alg5* and *dpg1* mutations both affect the synthesis of dolicholphosphoglucose (Runge *et al.*, 1984; Ballou *et al.*, 1986), whereas *alg6, alg8,* and *alg10* mutations seem to affect the specific glucosyltransferases directly. These mutations result in an accumulation of lipid-linked $Man_9GlcNAc_2$ (*alg6*; Runge *et al.*, 1984), $Glc_1Man_9GlcNAc_2$ (*alg8*; Runge and Robbins, 1986; Stagljar *et al.*, 1994), or $Glc_2Man_9GlcNAc_2$ (*alg10*; Zufferey *et al.*, 1995; Burda *et al.*, manuscript in preparation). All these mutations do not influence the growth of yeast but lead to hypoglycosylation of proteins. In combination with a mutation affecting oligosaccharyl transferase activity, a synthetic growth phenotype can be attributed to these mutations, thereby allowing the isolation of the corresponding wild-type loci by functional complementation in yeast (Stagljar *et al.*, 1994; te Heesen *et al.*, 1994).

## Results

### *Isolation of the ALG6 locus*

Our strategy to isolate the *ALG6* locus was based on the previous observation that mutations affecting the assembly of the lipid-linked oligosaccharide have a synthetic lethal phenotype in combination with *wbp1* mutations. These mutations alter one subunit of the oligosaccharyl transferase complex and result in a reduced OTase activity (Stagljar *et al.*, 1994). In particular, *alg6-1 wbp1-1* double mutants do not grow at 30°C, a temperature permissive for both single mutants. Introduction of a plasmid carrying the *WBP1* locus will result in a strain able to grow at 37°C, whereas an *ALG6* carrying plasmid will allow growth at 30°C, a temperature still permissive for the *wbp1-1* single mutant. We transformed the *alg6-1 wbp1-1* strain with a plasmid library consisting of partially digested yeast chromosomal DNA integrated into the vector YEp352 (Hill *et al.*, 1986) and selected among 50,000 individual transformants 24 strains which were able to complement the ts-defect at 30°C but not at 37°C. Recovery of the plasmid DNA from four transformants, amplification in *E.coli* and retesting in yeast revealed two plasmids that were able to complement the *alg6-1* mutation. Both plasmids contained inserts with common DNA sequences. One of these plasmids, pALG6-4, was analyzed further. The complementing DNA harboured a single complete open reading frame encoding a polypeptide of 544 amino acid residues and a predicted molecular weight of 62.8 kDa. The sequence of the *ALG6* locus is present in public databases (accession number U43491, ORF UNA544), identified in the course of the yeast genome sequencing project and is located

on chromosome XV (F.Sterky and M.Uhlen, unpublished observations)

*Complementation of the alg6-1 mutation by pALG6-4*

In order to confirm that the isolated locus not only complemented the growth defect of the *alg6-1 wbp1-1* double mutant at 30°C but also affected the glycosylation of secretory proteins, we analyzed the effect of the putative *ALG6* carrying plasmid on the glycosylation of the vacuolar carboxypeptidase Y (CPY) in different strains. Mature CPY contains four N-linked oligosaccharides (Hasilik and Tanner, 1978), but the correct targeting of CPY is not affected by a lack of oligosaccharides (Winther *et al.*, 1991). Mutations affecting the biosynthesis of the lipid-linked oligosaccharide or the activity of the oligosaccharyl transferase complex can lead to hypoglycosylation of CPY, resulting in distinct glycoforms of CPY (te Heesen *et al.*, 1992; Stagljar *et al.*, 1994) (Figure 2, lanes 2 and 3). The cumulative effect of the *alg6-1* and the *wbp1-1* mutations on protein glycosylation is visualized by the severe hypoglycosylation of CPY (Stagljar *et al.*, 1994) (Figure 2, lane 4). Introduction of the *WBP1* locus partially restores glycosylation of CPY, due to the complementation of the *wbp1-1* mutation (Figure 2, lane 5). Similarly, transformation of the *alg6-1 wbp1-1* double mutant with pALG6-4 reduced the hypoglycosylation of CPY (Figure 2, lane 6). We postulate that this partial suppression of hypoglycosylation is the cause for the suppression of the temperature sensitivity at 30°C. We also tested the complementation of the *alg6-1* single mutation by pALG6-4 (Figure 3). This plasmid is able to restore glycosylation of CPY in the *alg6-1* strain but not in the *wbp1-1* mutant

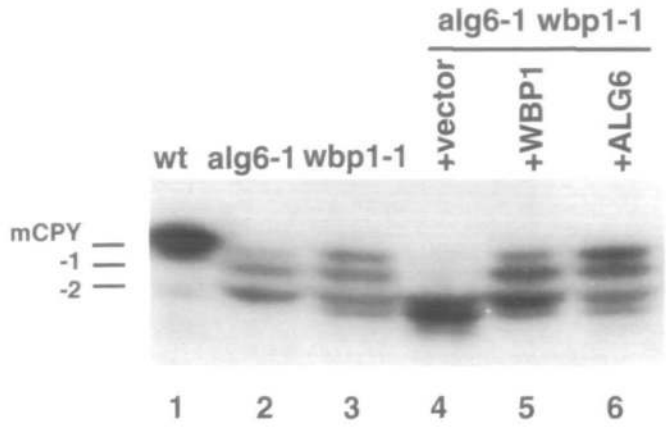


**Fig. 2.** Underglycosylation of CPY in the *alg6-1 wbp1-1* double mutant is partially complemented by either *ALG6* or *WBP1*. Strain YG36 was transformed with YEp352 (+vector), YEp352(WBP1) (+WBP1), or pALG6-4 (+ALG6). Western blotting of these transformants and strains MA7-B (wbp1-1) and PYR103 (alg6-1) was performed as described. The positions of mature CPY (mCPY) and the glycoforms lacking one (−1) or 2 (−2) oligosaccharides are indicated.

(Figure 3, lanes 3 and 6). We conclude that pALG6-4 specifically complements the *alg6-1* mutation.

*pALG6-4 encodes the ALG6 locus*

In order to confirm that the isolated locus carried by pALG6-4 is *ALG6*, we created an inactive version of the putative *ALG6* locus by replacing a DNA fragment encoding a large part of the

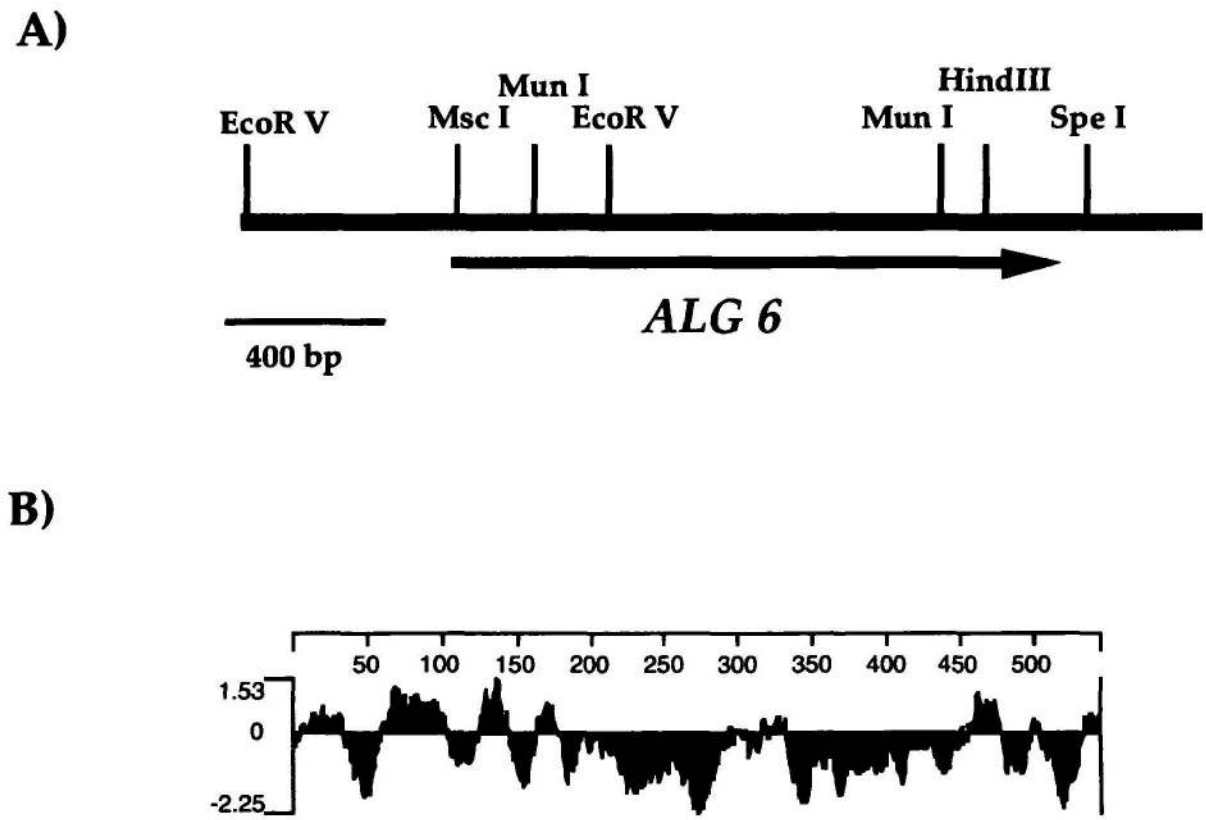


**Fig. 1.** (**A**) Restriction map of the *ALG6* locus. The cleavage sites for selected restriction enzymes and the location of the ORF are shown. (**B**) Hydropathy analysis of ALG6p according to the Kyte–Doolittle method (window of 19 residues) (Kyte and Doolittle, 1982).

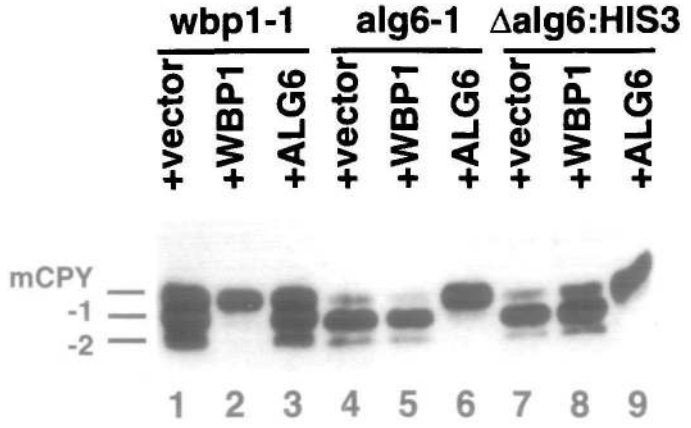


**Fig. 3.** pALG6-4 complements underglycosylation of CPY in *alg6-1* and Δ*alg6*, but not *wbp1-1* strains. Strains MA7-B (*wbp1-1*), PYR103(*alg6-1*), and YG227(Δ*alg6*) were transformed with YEp352 (+vector), YEp352(WBP1) (+WBP1), and pALG6-4 (+ALG6). Western blotting was performed as described. The positions of mature CPY (mCPY) and the glycoforms lacking one (−1) or 2 (−2) oligosaccharides are indicated.

ORF by the *HIS3* locus. This construct was used to replace one copy of the *ALG6* locus in a diploid strain by site directed integration. Correct integration was verified by PCR analysis (data not shown). Sporulation and subsequent analysis of the resulting tetrads revealed that a deletion of the isolated *ALG6* locus did not result in a detectable growth phenotype. However, when the glycosylation of CPY was analyzed in a Δ*alg6::HIS3* strain, we observed a similar hypoglycosylation as in the *alg6-1* strain (Figure 3, lanes 4 and 7). This glycosylation deficiency was complemented by expression of the wild-type *ALG6* but not the *WBP1* locus. The small difference observed in the glycosylation of CPY in *alg6-1* cells between Figure 2 (lane 2) and Figure 3 (lanes 4 and 5) might be due to different growth conditions used in the two experiments (23°C vs. 30°C).

The *alg6-1* mutation leads to the accumulation of incomplete lipid-linked oligosaccharide, namely $Man_9GlcNAc_2$ (Runge *et al.*, 1984). We therefore asked whether the deletion of the newly identified locus resulted in the same phenotype. Lipid-linked oligosaccharides were isolated from the original *alg6-1* and the Δ*alg6* strain, separated by HPLC chromatography, and compared to the oligosaccharides recovered from either wild-type or a Δ*alg5* strain (te Heesen *et al.*, 1994). The Δ*alg5* mutation inactivates Dol-P-Glc synthase and thereby promotes accumulation of lipid-linked $Man_9GlcNAc_2$ (Runge *et al.*, 1984; te Heesen *et al.*, 1994). Both the *alg6-1* and the Δ*alg6* mutation lead to an accumulation of a lipid-linked oligosaccharide comigrating with the $Man_9GlcNAc_2$ oligosaccharide in the Δ*alg5* strain (Figure 4).

The deletion in the isolated *ALG6* locus and the *alg6-1* mutation both resulted in the same phenotypes as visualized by CPY; underglycosylation and accumulation of incomplete lipid-linked oligosaccharide precursor ($Man_9GlcNAc_2$). From these data we conclude that we have isolated the *ALG6* locus. In support of this conclusion we found that both the *alg6-1* mutation and the artificially created Δ*alg6* allele map genetically to the same locus (data not shown).

### *The ALG6 locus encodes a protein similar to Alg8p*

The *ALG6* ORF encodes a putative protein of 544 amino acid residues which is highly hydrophobic (Figure 1) and has an

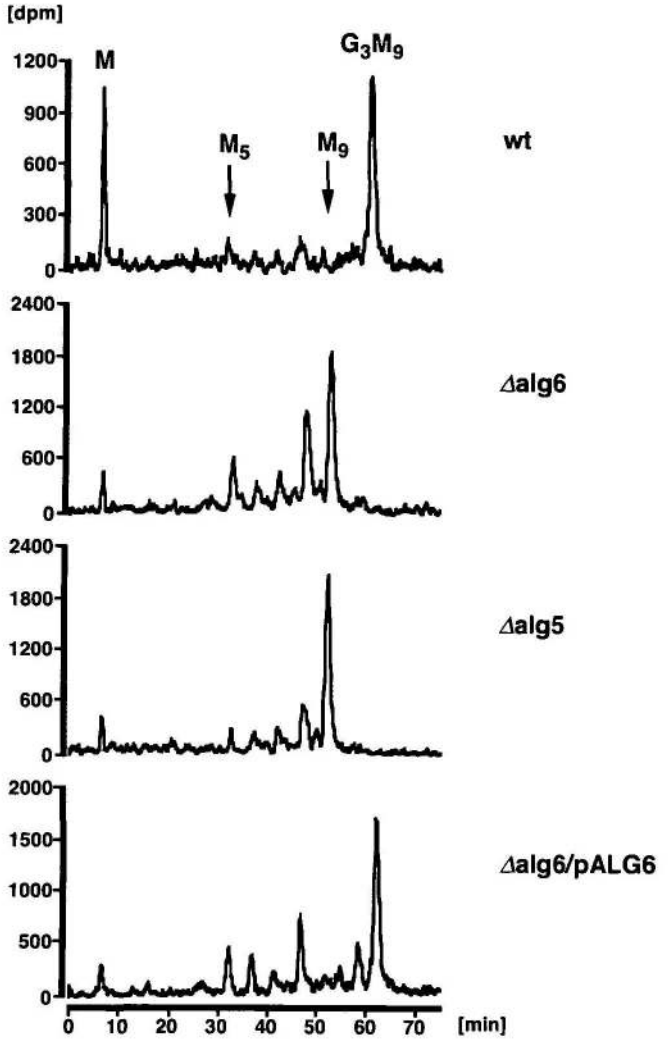


**Fig. 4.** Deletion of *ALG6* leads to the accumulation of incomplete lipid-linked oligosaccharide lacking the terminal glucose residues. Cells from strain YG227(Δ*alg6*) or YG227 transformed with pALG6-4 were labeled with [$^3$H] mannose. Lipid-linked oligosaccharides were extracted, hydrolyzed, and analyzed by HPLC chromatography. Oligosaccharides isolated from strains SS328 (wt) andYG91(Δalg5) served as standards. The position of $Glc_3Man_9GlcNAc_2$ ($G_3M_9$), $Man_9GlcNAc_2$ ($M_9$) and $Man_5GlcNAc_2$ ($M_5$) are indicated.

extremely high isoeletric point of 9.16. Two potential N-linked glycosylation sites (positions 170 and 441, Figure 5) were found. Due to the highly hydrophobic amino acid composition, several potential membrane spanning domains can be predicted (Persson and Argos, 1994). The lack of a cleavable signal sequence suggests that Alg6p is a type II transmembrane protein with multiple membrane spanning domains. Most interestingly, Alg6p is homologous in sequence to the previously identified *ALG8* protein of yeast (Stagljar *et al.*, 1994) (Figure 5). This sequence similarity is not unexpected since both proteins are required for glucosylation reactions of the lipid-linked oligosaccharide. In addition, a search in the available databases revealed two putative *C.elegans* proteins highly similar to both Alg6p and Alg8p (Figure 5).

## Discussion

We have isolated the yeast *ALG6* locus by complementation of the synthetic phenotype imposed by the *alg6-1* mutation in

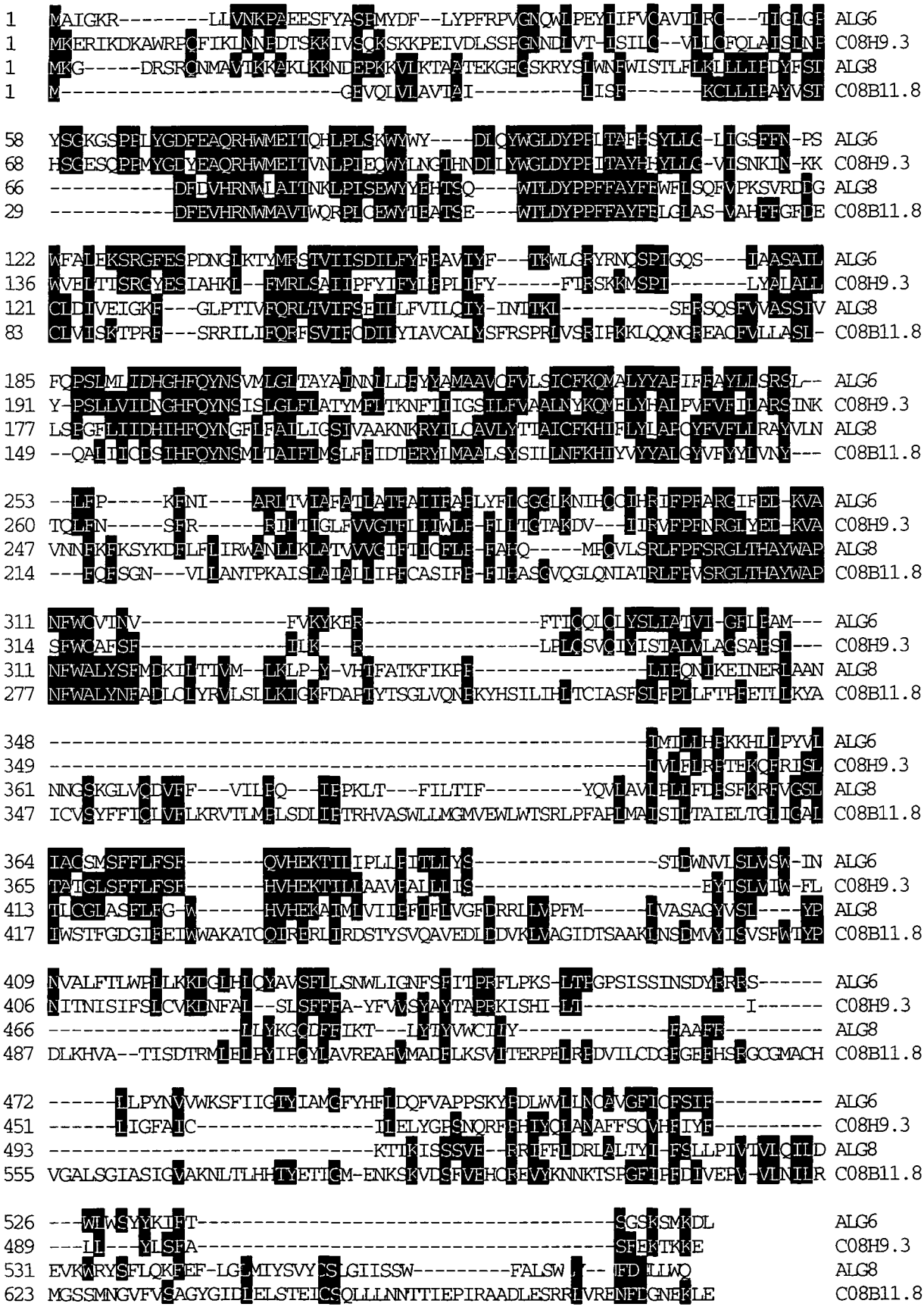


**Fig. 5.** Sequence identity between the primary amino acid sequence of ALG6p, ALG8p from *S.cerevisiae* and the putative *C.elegans* gene products C08H9.3 (accession number Z54342) and C08B11.8 (accession number Z46676). Identical amino acids in at least two of the four sequences are highlighted. The alignments were done using the DNASTAR software (Clustal method).

combination with the oligosaccharyl transferase mutation *wbp1-1*. The *alg6-1* mutation has been characterized previously (Runge *et al.,* 1984). Like the *alg5-1* mutation, it results in the accumulation of lipid-linked $Man_9GlcNAc_2$. However, the synthesis of the glucose donor Dol-P-Glc is not affected in the *alg6-1* strains. Runge *et al.* (1984) therefore concluded that the *ALG6* locus encodes the glucosyl transferase necessary for the transfer of the first α-1,3 glucose to the lipid-linked oligosaccharide. We have created a deletion in the *ALG6* locus and observed that this deletion lead to the same phenotype as did the previously identified *alg6-1* mutation. As also observed for other mutations affecting the addition of glucose residues to the lipid-linked oligosaccharide (Δ*alg5* and Δ*alg8*) (Stagljar *et al.,* 1994; te Heesen *et al.,* 1994), we note that this *alg6* deletion does not result in a growth defect. However, hypoglycosylation of secretory proteins was found in Δ*alg6* strains. This further supports the finding that the yeast oligosaccharyl transferase complex transfers incompletely glucosylated oligosaccharides to protein albeit with a reduced efficiency.

The *ALG6* protein is a highly hydrophobic polypeptide with multiple potential membrane spanning domains. It differs in this respect significantly from glycosyltransferases located in the Golgi compartment (Field and Wainwright, 1995), whereas at least one well characterized ER glycosyltransferase, UDP-Glc-NAc:dolichol phosphate N-acetylglucosamine-1-phosphate transferase, has been found to be an integral membrane protein (Zhu and Lehrman, 1990).

Sequence comparison reveals that it is similar in primary sequence (26% identity over 604 amino acid residues) as well as its hydrophobicity (Figure 5) to the *ALG8* polypeptide. This is not unexpected since both proteins encode putative glucosyltransferases which transfer glucose from the donor Dol-P-Glc onto the lipid-linked oligosaccharide, and both transferred glucose residues are α-1,3 linked to the lipid-linked oligosaccharide. Nevertheless, the two activities are highly specific because overexpression of Alg6p does not suppress the phenotypes imposed by a deletion in the *ALG8* locus or vice versa (G.Achleitner, S.te Heesen and M.Aebi, unpublished observations). These results demonstrate a substrate specificity of both the *ALG6* and the *ALG8* protein.

We observed that the newly identified *ALG6* protein is more closely related to a putative *C.elegans* protein than to the yeast Alg8p (Figure 5). This *C.elegans* protein was identified in the genome sequencing project (Wilson *et al.,* 1994), and therefore, no experimental evidence for its role in the biosynthesis of the lipid-linked oligosaccharide in this organism is available. We speculate that this protein is the Alg6p homologue of *C.elegans.* Yet another putative *C.elegans* protein was identified with a significant similarity to both the *ALG6* and the *ALG8* proteins of yeast. However, this protein is more similar to Alg8p than to Alg6p and we predict that this protein is the *C.elegans* homologue of Alg8p (Figure 5). Both the two yeast and the two *C.elegans* proteins are highly hydrophobic, but we note that the areas of highest primary sequence identity are in the rare hydrophilic domains of these polypeptides. In particular, the regions from amino acid positions 69 to 112, 187 to 201, and 296 to 314 (Alg6p sequence, Figure 5) are highly conserved. It is possible that these regions form the catalytic center for the transfer reaction which takes place in the hydrophilic environment. The same observation of high sequence conservation of hydrophilic domains in otherwise very hydrophobic proteins was also made for other lumenal ER glycosyltransferases (Aebi *et al.,* in press; Burda *et al.,* in press). This raises the question why these putative glucosyltransferases are so hydrophobic. Most likely, these hydrophobic domains are placed in the membrane and the reactions catalyzed by these lumenal glycosyltransferases involves two substrates anchored in the ER membrane by the lipid dolichol. It is possible that the hydrophobic areas are required for the binding of the dolichol moieties of these substrates.

## Materials and methods

### *Yeast strains and media*

Yeast strains used are listed in Table I. Standard yeast media were used (Guthrie and Fink, 1991).

### *Isolation of the ALG6 locus*

A genomic library (Stagljar *et al.,* 1994) containing partially digested yeast chromosomal DNA of 4–8 kb size ligated into the vector YEp352 was transformed into strain YG36 and transformants were selected on minimal medium lacking uracil at 23°C. Transformants that could grow as well at 30°C but not at 37°C were identified by replica-plating. Plasmid DNA from these colonies was recovered by extracting total yeast DNA which was used to transform *E.coli* strain DH5α to ampicillin resistance. Plasmids were tested by retransforming them into strain YG36.

### *Inactivation of the ALG6 locus*

A 1.3 kb *MunI/MscI* fragment was excised from *ALG6* coding region and replaced by the 1.8 kb *Bam*HI *HIS3* cassette (Struhl and Davis, 1981). The resulting plasmid was digested with *Eco*RV and *Spe*I to generate a 2.8 kb fragment directing integration at the *ALG6* locus. The purified fragment was used to transform strain SS328×SS330 to $His^+$. Correct integration was confirmed by whole cell PCR (Sathe *et al.,* 1991) using *ALG6* and *HIS3*-specific primers

### *Immunological methods*

Yeast cultures were grown over night at permissive temperature (23°C or 30°C) in either supplemented minimal or YPD medium. Cells were collected at log phase ($OD_{546}$ 1–1.5) by centrifugation. The amount of cells per sample corresponded to 4 OD units. Cells were suspended in 100 μl lysis buffer (50 mM Tris–HCl, pH 7.5, 1% SDS, 200 mM PMSF) and broken by the glass bead method as described previously (Franzusoff *et al.,* 1991). The final extract was mixed with 30 μl sample buffer (0.25 M Tris–HCl, pH 6.8, 8% SDS, 20% β-mercaptoethanol, 50% glycerol, 0.1% bromphenol blue) and boiled for 5 min. Samples (20–30 μl) were applied to 8% SDS–polyacrylamide gels and transferred onto nitrocellulose. Blots were probed for CPY with rabbit antiserum and visualized with peroxidase-labelled protein A using enhanced chemiluminescence (ECL, Amersham).

**Table I.** Yeast strains used in this study

| Strain | Genotype | Reference |
|---|---|---|
| SS328 | MATα ade2-101 ura3-52 his3Δ200 lys2-801 | te Heesen et al. (1992) |
| SS330 | MATa ade2-101 ura3-52 his3Δ200 tyr1 | te Heesen et al. (1992) |
| MA7-B | MATa ade2-101 ura3-52 his3Δ200 lys2-801 wbp1-1 | te Heesen et al. (1992) |
| PRY 103 | MATa ade2-101 ura3-52 alg6-1 | Runge et al. (1984) |
| YG36 | MATa ade2-101 ura3-52 his3Δ200 alg6-1 wbp1-1 | Stagljar et al. (1994) |
| YG227 | MATα ade2-101 ura3-52 his3Δ200 lys2-801 Δalg6::HIS3 | this study |
| YG91 | MATα ade2-101 ura3-52 his3Δ200 Δalg5::HIS3 | te Heesen et al. (1994) |

*Analysis of lipid-linked oligosaccharides*

Extraction and HPLC analysis of lipid-linked oligosaccharides was done as described (Zufferey *et al.*, 1995), except that the cells were grown in minimal medium supplemented with casamino acids (0.5%, Difco), adeninesulfate and tyrosine.

## Acknowledgements

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