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A screen for yeast mutants with defects in the dolichol-mediated pathway for N-glycosylation

(N-linked glycoproteins/dolichol biosynthesis/oligosaccharyl transferase)

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ABSTRACT **Dolichol in the form of dolichyl phosphate participates in the synthesis of N- and O-linked glycoproteins and phosphatidylinositol-linked proteins in the yeast *Saccharomyces cerevisiae*. In this organism, as well as in higher eukaryotes, a number of the enzymes in the polyisoprenoid and glycoprotein biosynthetic pathways have not been identified. In this study, we have developed a convenient, highly sensitive assay that uses one of the end products of the dolichyl-phosphate synthetic pathway, oligosaccharide-diphosphodolichol, and a ^{125}I-labeled peptide substrate for N-linked glycosylation to screen a collection of temperature-sensitive yeast mutants for defects in protein glycosylation. By using a combination of biochemical and genetic procedures, the defective mutants were grouped into three categories: those containing defects in dolichyl-phosphate synthesis (class 1), lipid-linked oligosaccharide assembly (class 2), or oligosaccharide transferase activity (class 3). Among the mutants identified by this screen were *sec59* (which encodes dolichol kinase) and a mutant that affects the activity of the *ALG1*-encoded mannosyltransferase that forms dolichol-*PP*-(GlcNAc)$_2$Man$_1$. Of particular interest was a mutant that exhibits a temperature-sensitive defect in oligosaccharide transferase activity. This mutant, *meg1* (microsomal protein essential for glycosylation 1) assembles a complete oligosaccharide chain and, therefore, is likely to be a class 3 mutant. We report the cloning of *MEG1*, the gene that rescues the oligosaccharide transferase activity defect in this mutant. A number of criteria distinguish this gene from previously described genes in this pathway.**

Since the discovery of the involvement of the long chain polyprenyl phosphate, dolichyl phosphate, in the pathway of assembly of the oligosaccharide chain of N-linked glycoproteins (for reviews, see refs. 1 and 2), a great deal of progress has been made in understanding the individual steps of this complex process. As shown in Fig. 1, the overall pathway can be considered to occur in three phases. A number of the early steps in the first phase, the synthesis of dolichyl phosphate, have been elucidated. However, the details of the late steps in formation of this lipid, as well as the regulation of its synthesis, are not well understood. The second phase is the multistep, lipid-linked oligosaccharide assembly process, whereby dolichyl phosphate serves as an anchor for the assembly of oligosaccharide-disphosphodolichol. As shown, numerous steps in this phase have been delineated in yeast as the result of the availability of *alg* mutants (asparagine-linked glycosylation mutants; ref. 3), although, here too, the details of some steps and the topology of the enzymes and products are unclear. Characterization of the enzyme involved in the final step, in which the oligosaccharide chain is transferred from the lipid anchor to -Asn-Xaa-Ser/Thr- sites in proteins has, for a number of year, eluded investigators. This step, catalyzed by oligosaccharide transferase (OT), has recently been characterized in part by Gilmore and coworkers (4, 5), who reported that in canine pancreas microsomes OT consists of a three-component complex. Similarly, studies in yeast have provided evidence that OT in yeast contains at least two protein components (6, 7).

As an outgrowth of earlier efforts to determine the specificity of OT we prepared a variety of labeled -Asn-Xaa-Ser/Thr- peptides to determine their efficiency as substrates. The most effective substrate was found to be Asn-Lys(N^{ε}-*p*-azidobenzoyl)-Thr-NH_2 whose amino terminus was derivatized with ^{125}I-labeled Bolton–Hunter reagent (bh) (8). The availability of this peptide substrate enabled us to develop a simple, sensitive assay that could be used on small cultures of lysed yeast protoplasts. The objective was to identify yeast mutants that exhibited defects in one of the three phases of the dolichol-linked pathway: synthesis of dolichyl phosphate, assembly of oligosaccharide-diphosphodolichol, or transfer of the complete oligosaccharide to the acceptor protein or peptide. As is evident in Fig. 1, a defect leading to underproduction of any of the intermediates in phase 1 or 2 would result in impaired peptide glycosylation in phase 3.

In this study we report the results of the use of this assay as a screen for such yeast mutants and its application to a collection of temperature-sensitive (ts) mutants (9). The validity of the screen is documented by the identification of known mutants in phases 1 and 2 of the overall assembly process (class 1 and class 2 mutants). In addition, evidence for the identification of a class 3 mutant that exhibits a ts defect in OT activity is reported. *MEG1*, the gene that rescues the activity defect of this mutant, has been cloned. A number of criteria, including partial DNA sequence analysis, suggests that *MEG1* is an additional component of the OT complex.

EXPERIMENTAL PROCEDURES

Strains. The *Saccharomyces cerevisiae* ts collection of mutants isolated by Hartwell (9) was used in this study. The parent strain from which the ts collection was derived is A364A (*MATα ade1 ade2 ura1 lys2 tyr1 his7 gal1*). Strains RS868 (*MATα ade1 ade2 his3 his7 leu2 trp1 ura3*) and W303-1b (*MATα ade2 can1 his3 leu2 trp1 ura3*) were used for genetic crosses with the mutants. Strains PRY55 (*MATα alg1-1 ura3-52*), PRY212 (*MATα alg2-1 ura3-52*), and PRY95 (*MATα alg4-4 ura3-52*) were obtained from P. Robbins (Department of Biology, Massachusetts Institute of Technology). RSY27 (*MATα sec59-1 his4-593 ura3-52 suc2-432*) was obtained from R. Schekman (Department of Biochemistry, University of California-Berkeley).

Plasmids and Vectors. pJR234 (the *Bam*HI fragment of the *ALG7* genomic clone inserted into pBR322) was obtained from J. Rine (Department of Biochemistry, University of

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Abbreviations: OT, oligosaccharide transferase; bh, Bolton–Hunter reagent; ts, temperature sensitive; wt, wild type.

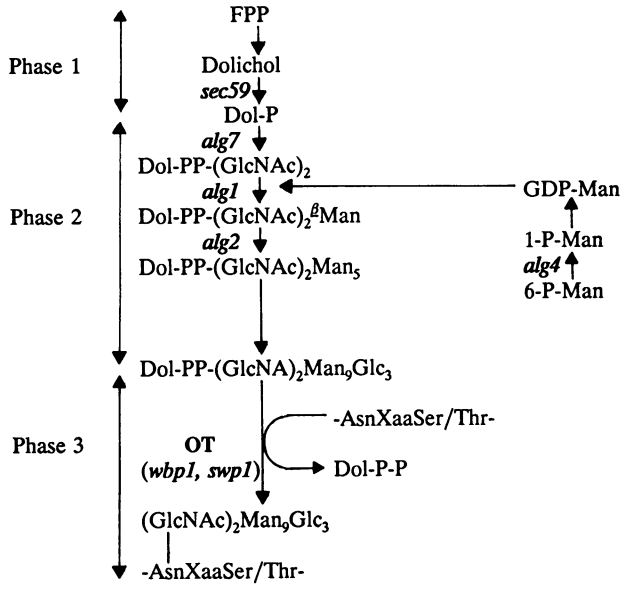

FIG. 1. The glycosylation pathway. Yeast mutants known to affect various steps in the pathway are indicated (ts strains of *sec59*, *alg1*, *alg2*, *alg4*, and *wbp1* have been isolated). FPP, farnesyl-*PP*; Dol, dolichol.

California, Berkeley). pWBP1 (encoding a truncated form of *WBP1*) and pSWP1 (the *Pst* I–*Bam*HI fragment of the *SWP1* genomic clone inserted into YEp352) were obtained from M. Aebi (Institute for Molecular Biology, University of Zürich). pSEC5920 was obtained from R. Schekman. Plasmids pSEYc68 (S. Emr, University of California San Diego) and p366 (P. Hieter, Johns Hopkins School of Medicine) were used as cloning vectors, and pBluescript (Strategene) was used for cloning and sequencing.

General Methods and Materials. Standard protocols (10) were followed for media preparation, sporulation, and tetrad dissection. Yeast microsomes were prepared as described by Rothblatt and Meyer (11), except that cells were grown at 23°C instead of 30°C. Yeast transformations were carried out by the lithium acetate procedure (12), enhanced by the addition of dimethyl sulfoxide (13), but with the following modifications: cells were grown at 23°C and heat shocked at 37°C for 5 min. Complementation analysis between two ts mutants was done by assaying the appropriate diploids for growth at 36°C. All restriction enzymes were purchased from Boehringer Mannheim. Restriction digests and analysis were done as described by Sambrook *et al.* (14). All other reagents used were of the highest grade available from either Difco, Fisher Scientific, or Sigma.

Asn-Lys(N^{ε}-*p*-azidobenzoyl)-Thr-NH_2 was a generous gift of S. Khan (Wistar Institute). The amino terminus was derivatized with ^{125}I-labeled bh (monoiodo form, 2000Ci/mmol; 1 Ci = 37 GBq; Amersham) as described (15), yielding ^{125}I-bh-Asn-Lys(BzN_3)-Thr-NH_2, which was then purified by HPLC on a C_{18} reverse-phase column (Vydac) using a 0–60% acetonitrile gradient (16).

Cloning of *MEG1*. Mutant 163 was transformed with a yeast genomic library in a centromere-containing vector marked with *LEU2*. This library, obtained from F. Spencer and P. Hieter, consists of yeast genomic DNA partially digested with *Sau*3A and inserted at the *Bam*HI site of vector p366, a YCp50 derivative in which *URA3* is replaced by *LEU2*. Transformants were screened for ability to grow at 36°C. Plasmids were isolated by electroporation of *Escherichia coli* with yeast miniprep DNA (17).

OT Activity Assay. Yeast strains were grown overnight in YPD medium at 23°C to midlogarithmic phase. Cultures were then divided in two; one aliquot was incubated at 36°C for 90 min, and the other remained at 23°C. Cells were collected and then resuspended in spheroplasting buffer (1.2 M sorbitol/20 mM potassium phosphate, pH 7.4/1 mM 2-mercaptoethanol). Zymolyase-100T (ICN) was then added and allowed to incubate for 30 min at 23°C. Spheroplasted cells were collected and resuspended in glycosylation buffer (50 mM Tris·HCl, pH 7.4/10 mM $MnCl_2$/1 mM 2-mercaptoethanol). ^{125}I-bh-Asn-Lys(BzN_3)-Thr-NH_2 peptide was then added, and the reaction proceeded for 20 min at 23°C. The reaction was stopped by addition of Nonidet P-40 (Calbiochem) to a final concentration of 1%. Glycopeptide formation was then quantitated by the binding of the glycopeptide to ConA-agarose beads (Sigma) and counted in a γ counter (Wallac/Pharmacia LKB, model 1275 Minigamma).

Glycopeptide formation by microsomes was done as described (18). Biochemical complementation experiments (see *Results*) used the microsomal OT activity assay supplemented with 125 μM dolichyl phosphate, 25 μM UDP-Glc, 25 μM GDP-Man, and 100 μM UDP-GlcNAc (all purchased from Sigma) and were performed in the presence of 0.075% octyl β-glucoside (Boehringer Mannheim).

Characterization of *in Vivo*-Labeled Oligosaccharides. Cells were labeled with [^{3}H]mannose (25 Ci/mmol; ICN Biochemicals) as described (19) except that the mild acid hydrolysis treatment was performed as reported by Rush *et al.* (20). The hydrolysate was then dried down, resuspended in H_2O, extracted twice with an equal volume of diethyl ether, and applied to a Bio-Gel P-4 column (minus 400 mesh, 1.5 cm × 105 cm). Fractions of 1 ml were collected. The V_o (blue dextran) was determined to be 60 ml, and the V_i ([^{3}H]mannose) was determined to be 150 ml.

RESULTS AND DISCUSSION

Identification of ts Mutants Defective in Glycopeptide Formation. The initial objective of this screen was to identify yeast mutants defective in OT activity. The simple assay employed is extremely sensitive due to the high affinity of the enzyme for the peptide substrate and the very high specific radioactivity of the peptide (8, 21). Moreover, quantitation of labeled glycopeptide formation by use of ConA-agarose beads that bind α-mannosyl residues is highly specific. Of the 440 ts strains in the Hartwell collection available to us, 285 were assayed as described in *Experimental Procedures*. Fifteen mutants were found to exhibit either a significant (>30%) reduction in peptide glycosylation upon shifting to the nonpermissive temperature (36°C) or had <50% of the level of peptide glycosylation of the parental strain at 23°C. The degree of impairment of peptide glycosylation in lysates of 7 of these mutants is shown in Table 1; the remaining 8 mutants remain to be characterized.

The first step in characterization of these mutants was to determine if the reduction in peptide glycosylation was the result of a lesion in an endoplasmic reticulum associated protein rather than a mutation elsewhere in the cell. To do this, microsomes were prepared from each mutant, and equivalent amounts of microsomal protein were assayed for peptide glycosylation. The results of this assay, also shown in Table 1, indicate that six of the seven mutants exhibited reduced levels of glycosylation activity at the level of microsomes. Microsomes from mutant 212 could not be prepared because this strain did not spheroplast under the conditions used in both the screen and in the microsome preparation. Thus, the apparent lack of glycosylation activity in lysates of mutant 212 was probably a result of the failure to deliver the peptide substrate to the cell.

The second step in characterizing the remaining six mutants was to determine if the defect in peptide glycosylation activity was the result of a mutation in one gene and, if so, to

Table 1. Peptide glycosylation activity

Mutant	Peptide glycosylation, % wt at 23°C		
	In lysates*		In microsomes†
	23°C	36°C	23°C
A364A (wt)	100	100	100
59	24	43	23
64	37	71	31
148	46	61	14
163	9	9	20
212	1	1	n.a.
265	19	29	16
283	64	19	18

NA, not analyzed.
*The assay is described in *Experimental Procedures*. The average value for A364A (wt) was 90,000 cpm, and that for mutant 163 was 8000 cpm.
†One hundred fifty micrograms of microsomal protein was assayed.

determine if the mutation gave rise to the ts growth phenotype. Mutants were mated with wild-type (wt) strains (see *Experimental Procedures*) and sporulated, and the resulting tetrads were dissected (an average of six tetrads per mutant were examined). Segregants from each full tetrad were then assayed for peptide glycosylation activity and growth at 36°C. The depressed level of peptide glycosylation from five of the six mutants (59, 148, 163, 265, and 283) was found to segregate 2:2 with respect to wt activity, showing that in these cases the peptide glycosylation defect was due to a single nuclear mutation. Additionally, the glycosylation defects in mutants 163 and 283 were found to cosegregate with the ts growth phenotype (data not shown).

Three Classes of Mutants Can Be Identified by This Screen. As shown in Fig. 1 and discussed in the Introduction, starting from the first step committed to dolichol synthesis (elongation of farnesyl-*PP*), a mutation detected by the screen measuring impairment of peptide glycosylation may fall into one of three classes corresponding to the three phases in the assembly process. Any mutation in the dolichol synthesis pathway (class 1) will lead to a decrease in the endogenous pool of lipid-linked oligosaccharide and, therefore, a decrease in peptide glycosylation. Similarly, mutations in enzymes involved in the assembly of the lipid-linked oligosaccharide (class 2), such as the asparagine-linked glycosylation, or *alg* mutants (3), will exhibit a decrease in peptide glycosylation. Parenthetically, only those class 2 mutants with alterations early in the oligosaccharide assembly pathway will be detected in this screen because of the specificity of the ConA-agarose beads. Finally, class 3 mutants are those that will exhibit impaired peptide glycosylation not because of a defect in the assembly of oligosaccharide-diphosphodolichol but as the result of an actual lesion in any of the components of OT.

Mutants 64 and 283 Are Class 1 Mutants. To distinguish mutants in the dolichol synthesis pathway (class 1) from all others, a biochemical complementation experiment was designed. In this experiment, dolichyl phosphate and UDP-GlcNAc, GDP-Man, and UDP-Glc were incubated with microsomes prepared from each mutant. Any mutant having a defective enzyme in the dolichol synthesis pathway, and, therefore, a reduced pool of endogenous dolichyl phosphate to serve as a substrate in lipid-linked oligosaccharide assembly, will be rescued by the addition of exogenous dolichyl phosphate and the appropriate sugar nucleotides. The results in Table 2 reveal that the low activity exhibited by microsomes prepared from mutants 64 and 283 is completely restored by supplementation with exogenous dolichyl phosphate and sugar nucleotides. Thus, mutants 64 and 283 are class 1 mutants.

Table 2. Effect of exogenous dolichyl phosphate and sugar nucleotides on peptide glycosylation in microsomes of mutants

Mutant	Peptide glycosylation activity in microsomes, % wt at 23°C	
	No addition	+ Dol-*P* and NDP-glycoses
A364A (wt)	100	120
59	23	30
64	31	120
163	23	23
265	16	15
283	18	130

Dol, dolichol.

Many of the steps in the lipid-linked oligosaccharide assembly pathway are known to be catalyzed by essential genes in yeast, and strains possessing ts mutations in these genes have been isolated (Fig. 1; for reviews, see refs. 2 and 22). As mentioned previously, in mutant 283 the temperature sensitivity and glycosylation defect were found to cosegregate. *sec59* (a ts mutant defective in dolichol kinase activity) is the only class 1 ts strain isolated to date; therefore, mutant 283 was crossed with *sec59* to ascertain if mutant 283 was allelic to *sec59*. Complementation analysis revealed that mutant 283 failed to complement *sec59*. This finding, in conjunction with the biochemical complementation data, strongly suggested that mutant 283 is allelic to *sec59*. To confirm this observation, mutant 283 was transformed with pSEC5920, a plasmid carrying the wt *SEC59* gene, and transformants were then assayed for peptide glycosylation activity. The result of this experiment, shown in Table 3, is that the activity defect in mutant 283 is rescued by transformation with the gene encoding dolichol kinase.

Mutant 148 Is a Class 2 Mutant. Mutants 59, 64, 148, and 265 exhibit glycosylation defects that were not directly linked to the temperature sensitivity. It remains to be established if these lesions result from either leaky mutations in essential genes or mutations in nonessential genes. Interestingly, when mutants 59, 148, and 265 were crossed with the *alg1*, *alg2*, and *alg4* ts strains and assayed for growth at 36°C, mutant 148 did not complement an *alg1* ts strain. Thus, mutant 148 may have a leaky mutation in the *ALG1* gene or may exhibit a genetic interaction with the *ALG1* gene product. In either event, on this basis, mutant 148 can be classified as a class 2 mutant.

Mutant 163 Is a Class 3 Mutant. In mutant 163, as mentioned above, the temperature sensitivity and glycosylation defect were found to cosegregate. The depressed peptide glycosylation activity in mutant 163 microsomes was not rescued by addition of exogenous dolichyl phosphate, and mutant 163 complemented the *sec59* strain. Consequently mutant 163 is not a class 1 mutant. Moreover, mutant 163 complemented three of the known ts strains (*alg1*, *alg2*, and *alg4*) defective in the lipid-linked oligosaccharide assembly pathway, indicating that it was not defective in any of these known steps in this process. To distinguish if mutant 163 was an additional class 2 mutant or a class 3 mutant, the lipid-linked oligosaccharide synthesized by mutant 163 was char-

Table 3. Glycosylation activity of mutant 283 is rescued by transformation with the gene encoding dolichol kinase (*SEC59*)

Mutant	Relative peptide glycosylation	
	23°C	36°C
A364A	100	100
283	57	24
283 (pSEC5920)	98	90

acterized. If the lesion in mutant 163 was in OT, then this mutant would be expected to synthesize wt oligosaccharide-diphosphodolichol [dolichol-*PP*-$(GlcNAc)_2Man_9Glc_3$], whereas a class 2 lesion would result in a truncated form of the oligosaccharide chain (3, 19). When wt cells were labeled with [^{3}H]mannose at 36°C and the oligosaccharide chain of the lipid-linked oligosaccharide was analyzed, the major component was the complete oligosaccharide, $Glc_3Man_9(GlcNAc)_2$; intermediate-sized oligosaccharides could also be detected (Fig. 2*A*). Mutant 163 also synthesized the complete oligosaccharide at the same level as wt (Fig. 2*B*), as well as shorter oligosaccharides. The analysis of the *alg2* oligosaccharide shown in Fig. 2*C* demonstrates that this method can detect alterations in the size or amount of the complete oligosaccharide chain attached to lipid since, as expected, little or no complete oligosaccharide was detected in this strain at 36°C. These data clearly establish that the lesion in mutant 163 is not in the assembly of the oligosaccharide linked to dolichol.

Mutant 163 Is Rescued by pMEG1. Because mutant 163 was a strong candidate to be a class 3 mutant (i.e., defective in OT), it was important to clone the wt gene. Accordingly, mutant 163 was transformed with a single-copy, centromere-containing yeast genomic library. Transformants were selected at 23°C on selective media plates and then replica plated and selected for growth at 36°C. Of 8000 transformants

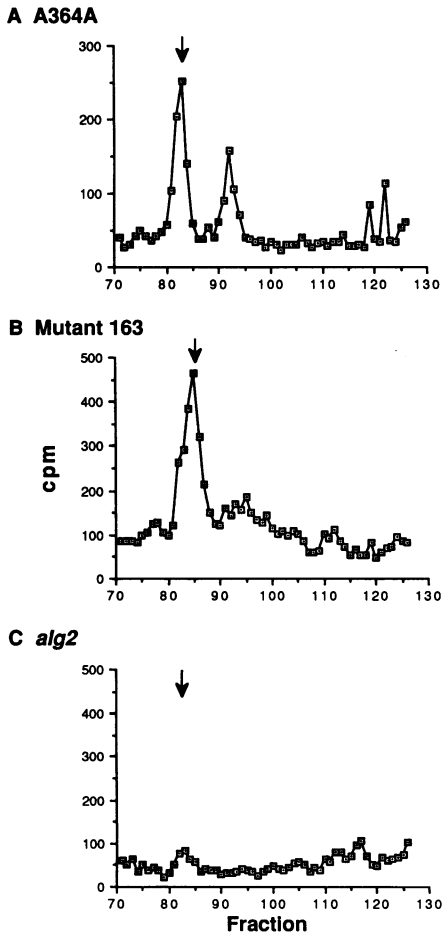

FIG. 2. Characterization of the lipid-linked oligosaccharides synthesized *in vivo* by A364A (wt) (*A*), mutant 163 (*B*), and *alg2* (*C*). Cell cultures were incubated with [^{3}H]mannose at 36°C. Lipid-linked oligosaccharides were extracted and hydrolyzed, and the resulting oligosaccharides were analyzed by gel-filtration chromatography (Bio-Gel P4, minus 400 mesh). One-half of each labeling reaction was loaded onto the column. The arrow denotes the peak fraction containing the complete oligosaccharide [$(GlcNAc)_2Man_9Glc_3$].

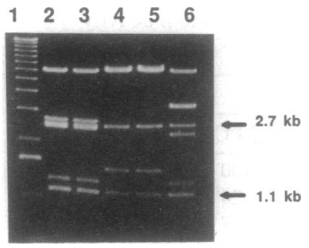

FIG. 3. Restriction analysis of mutant 163 transforming plasmids. Plasmids from transformants that grew at 36°C were isolated, electroporated into *E. coli*, and reisolated from the bacteria. These plasmids were then digested with *Eco*RI and analyzed by agarose gel electrophoresis. Lane 1, 1-kb DNA ladder; lanes 2 and 3, pMEG1.1; lanes 4 and 5, pMEG1.2; lane 6, pMEG1.3.

screened, 5 grew at 36°C. Plasmids from these transformants were isolated and electroporated into *E. coli*, and the DNA isolated from the bacteria was digested with *Eco*RI. The digests were then analyzed by agarose gel electrophoresis. The results shown in Fig. 3 revealed that the 5 transformants were rescued by three different plasmids, but all three possessed common 1.1-kb and 2.7-kb *Eco*RI fragments. Thus, one gene, situated within three overlapping partial digests of yeast genomic DNA, rescues the ts phenotype of mutant 163. This gene either encodes a subunit of OT or a component that affects the activity of this enzyme. The gene, named *MEG1* for *m*icrosomal protein *e*ssential for *g*lycosylation *1*, is situated within the plasmids designated pMEG1.1–pMEG1.3. Transformation for mutant 163 with pMEG1.1 generated transformants that grew at 36°C and exhibited wild-type OT activity (Table 4). The plasmid pMEG1.2 was subcloned as shown in Fig. 4. Subclones were tested for the presence of the nutritional marker and then tested for complementation of the OT activity defect in mutant 163. As shown, pJR4, made up of a 4.2-kb *Eco*RI partial fragment derived from pMEG1.2 and cloned into pSEYc68, complemented the OT activity defect in mutant 163. Preliminary sequence analysis of pJR4 revealed no sequence homology to any protein already identified, including those implicated as components of OT [i.e., Wbp1p and Swp1p in yeast (6, 7) and OST48, ribophorin I, and ribophorin II in mammals (4, 5)]. Furthermore, restriction analysis of pMEG1 revealed that the restriction pattern of pMEG1 was distinct from that of the *ALG7*, *WBP1*, or *SWP1* gene (data not shown). Additionally, transformation for mutant 163 (*meg1*) with pSWP1, a multicopy suppressor of *WBP1* (7), did not rescue the temperature sensitivity of mutant 163 (data not shown).

In summary, we have designed a simple, yet highly sensitive assay to screen a ts collection of yeast strains for mutants defective in N-linked glycosylation. Initially, this screen was designed to identify components of OT. However, because this screen requires the presence of endogenous lipid-linked oligosaccharide, which is one of the two substrates for OT, it is clear that this screen can be used to identify defects in enzymes in the dolichol synthesis pathway, as well as lesions in assembly of oligosaccharyl-*PP*-dolichol (See Fig. 1). Biochemical complementation experiments can quickly identify mutants as either possessing a defect prior to or after the point of dolichyl phosphate

Table 4. Glycosylation activity of mutant 163 is rescued by transformation with pMEG1.1

Mutant	Relative OT activity
A364A	100
163	8
163 (pMEG1.1)	92

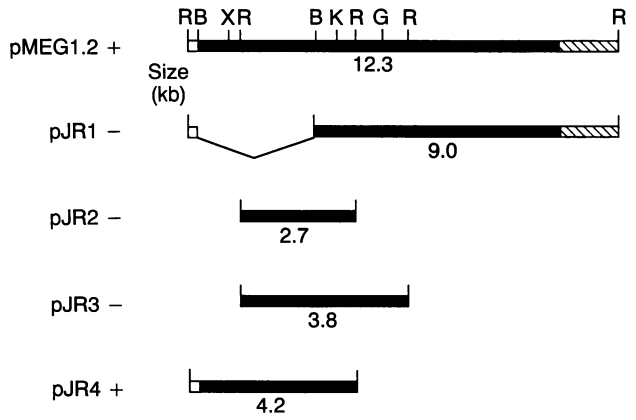

FIG. 4. Subcloning of *MEG1*. The DNA inserts of pMEG1.2 and of various constructed plasmids are shown (solid bars), aligned by their restriction sites. The inserts of pMEG1.2, pJR1, and pJR4 contain 375 bp of vector derived from p366 (open bars); pMEG1.2 and pJR1 contain an additional 2 kb of vector derived from the *LEU2* gene in p366 (hatched bars). After the name of each plasmid, "+" or "–" indicates complementation or noncomplementation, respectively, of mutant 163 by that plasmid. R, *Eco*RI; B, *Bam*HI; X, *Xho* I; G, *Bgl* II; K, *Kpn* I.

synthesis. More specifically, as shown in this study, addition of dolichyl phosphate and the three sugar nucleotides can overcome loss of glycosylation activity in microsomes as a result of a class 1 mutation. It will not, however, rescue class 2 or 3 mutants. Similarly, addition of exogenous oligosaccharyl-*PP*-dolichol with concomitant restoration of activity in microsomes would assign the mutant to be a class 1 or 2 mutant, whereas a failure to regain activity would point toward a defective class 3 mutation (i.e., OT). Alternatively, analysis of the oligosaccharide chain of the endogenous oligosaccharyl-*PP*-dolichol isolated from the mutant strain can distinguish between a defect in the oligosaccharide assembly pathway and a defective OT. These biochemical approaches, in conjunction with the use of yeast genetic techniques, should lead to the identification of the enzymes that catalyze steps in the pathway that are not well understood. These include (*i*) enzymes in the later steps in polyisoprenoid synthesis, whereby polyprenyl diphosphate is converted to dolichyl phosphate, (*ii*) enzymes in the assembly of oligosaccharyl-*PP*-dolichol as well as proteins that may be translocators or "flippases" of the lipid-linked oligosaccharide, and (*iii*) the enzyme that is the focus of our current efforts, OT.

With respect to OT, our finding provides further evidence that this enzyme in yeast is a multisubunit enzyme. Earlier work by Aebi and coworkers (6, 7) provided evidence that two proteins, Wbp1p and Swp1p, are components of this enzyme. Both proteins are essential for viability; yet, both are nonlimiting components of OT and are believed to be integral membrane proteins based on their deduced amino acid sequence. Using the screen described in this report, we have identified a ts mutant (163) in the Hartwell collection that is defective in peptide glycosylation. This defect is not a result of an alteration in the structure of the endogenous oligosaccharyl-*PP*-dolichol substrate, since analysis revealed that in the mutant the substrate is identical in size to the wt oligosaccharide. Genetic complementation studies, restriction mapping, and partial sequence analysis revealed that the mutation is not in a known gene in the oligosaccharyl-*PP*-dolichol assembly pathway or in *WBP1* or *SWP1*. Consequently it seems clear that this gene encodes a component of OT or a molecule that affects the activity of OT.

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