The OST4 Gene of Saccharomyces cerevisiae Encodes an Unusually Small Protein Required for Normal Levels of Oligosaccharyltransferase Activity*

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Sodium vanadate is an effective drug for the enrichment of yeast mutants defective in glycosylation reactions that are carried out in the Golgi complex (1). We have isolated vanadate-resistant, hygromycin B-sensitive mutants that act at very early steps of N-linked glycosylation, occurring in the endoplasmic reticulum. Here we describe the phenotypic characterization of ost4, a vanadate-resistant mutant that is defective in oligosaccharyltransferase (OTase) activity both in vivo and in vitro. The OST4 open reading frame is unusual in that it predicts a protein of only 36 amino acids. We demonstrate that the OST4 gene product is, in fact, an unusually small protein of approximately 3.6 kDa, predicted to lie almost entirely in the hydrophobic environment of the membrane. Strains carrying a disruption of the OST4 gene are viable but grow poorly at 25 °C. The null mutant is inviable at 37 °C, demonstrating that the OST4 gene product is essential for growth at high temperatures. Deletion of the OST4 gene greatly diminishes OTase activity but does not abolish it. These results suggest that the OST4 gene encodes a subunit or accessory component of OTase that is essential at high temperature.

Glycosylation is an important modification that regulates the structure and function of secreted and membrane proteins. The early steps of N-linked glycosylation are highly conserved among eukaryotes and begin in the endoplasmic reticulum. The assembly of a lipid-linked precursor oligosaccharide occurs through the ordered addition of sugars onto a dolichol phosphate anchor to generate Glc3Man9GlcNAc2-PP-dolichol (2, 3). The preassembled oligosaccharide is then transferred as a unit onto an appropriate asparagine residue in a nascent polypeptide. This transfer is catalyzed by the enzyme N-oligosaccharyltransferase (OTase). Recent progress in the characterization of this enzyme in yeast has been achieved by the convergence of both genetic and biochemical approaches. In Saccharomyces cerevisiae, five genes encoding components of OTase have been identified: WBP1 (4), SWP1 (5), OST1 (6), and OST3 (7). WBP1 is required for N-linked glycosylation in vivo and in vitro (4). SWP1 was isolated as an allele-specific high copy suppressor of a wbp1 mutant (5). Both genes encode predicted transmembrane proteins, which are localized in the endoplasmic reticulum and are essential for viability. The Wbp1 protein can be cross-linked to the Swp1 protein in extracts of microsomal membranes, suggesting that these proteins form a complex in vivo (5). Yeast OTase activity co-purifies with a protein complex of six major subunits, termed α (64 kDa), β (48 kDa), γ (34 kDa), δ (30 kDa), ε (16 kDa), and θ (9 kDa) (6). Among these, the β and δ subunits were shown to correspond to the 48-kDa Wbp1 and the 30-kDa Swp1 proteins, demonstrating that Wbp1 and Swp1 are indeed structural components of OTase (8, 9). The OST1 gene, a homolog of mammalian ribophorin I, encodes the 64-kDa α subunit gene and is also an essential gene product (6). Ost3p corresponds to the 34-kDa γ subunit. Unlike the other subunits, this protein is not required for viability, suggesting that it functions as an auxiliary component of the OTase complex (7). A fifth yeast mutant, m163, has been identified that is defective in OTase activity in vitro (10). An extragenic suppressor of m163 has been identified, but the wild-type gene defined by this mutant has not been isolated.

We have isolated yeast mutants that are defective in glycosylation, based upon resistance to sodium vanadate (1). Among these, we found two mutants with defects in steps that affect early N-linked glycosylation. These mutants identify OST4, a novel gene that encodes an unusually small protein required for OTase activity in vivo and in vitro. Our ost4 mutants are allelic to m163.

MATERIALS AND METHODS

Strains and Media—The yeast strains used in this study are listed in Table I. Yeast strains were grown in either YPAD (1% yeast extract, 2% peptone, 2% dextrose, 50 mg/liter adenine sulfate) or synthetic media that contained 0.67% yeast nitrogen base and 2% glucose, supplemented with the appropriate auxotrophic requirements (11). YPAD was supplemented with 0.5 M KCl for the growth of all vanadate-resistant mutants. Sodium ortho-vanadate (Fisher) was freshly prepared as a 1.0 M solution in sterile water and added to autoclaved YPAD+ agar to a final concentration of 7–10 mM just prior to pouring plates. Hygromycin B (Boeringer Mannheim) was added to autoclaved YPAD + agar to a final concentration of 30 μg/ml, just prior to pouring plates.

Plasmids—Standard methods were used for all cloning (12). pPOST4R1 contains a 1086-base pair EcoRI fragment cloned into the yeast shuttle vector, pRS316 (13). All of the following plasmids, used for complementation analyses (see Fig. 4) are subclones derived from this EcoRI fragment, cloned in pRS316. pHC1 contains a 940-base pair EcoRI/HindIII fragment; pHC2 contains a 446-base pair HindIII/EcoRI fragment; pHC3 contains a 357-base pair HindIII/XhoI fragment; pHC4 contains a 275-base pair HindIII/SalI fragment; pHC5 was constructed by digesting pPOST4R1, containing the entire 1086-base pair EcoRI fragment with StyI, leaving a four-base 5′ overhang. This overhang was then filled in with Klenow, and the plasmid was reli-
gated, resulting in an insertion of four base pairs to create a frameshift; all but the last 24 base pairs of the coding sequence with the recognition sequence for N-linked oligosaccharide addition, was prepared by iodinating Asn-Lys(Thr) or Thr-.NH2 with 125I-labeled Bolton-Hunter reagent as described previously (10). The disruption plasmid, post4:URA3, was constructed by replacing the HindIII/HindI fragment in pOST4Rl with a Sall/Smal fragment containing the entire URA3 gene (filled in with Klenow), thereby replacing all but the last 24 base pairs of the OST4 coding sequence with the URA3 gene.

**Gated Methods—**Standard genetic methods for complementation analysis, tetrad analysis, and mating of haploid cells were employed (14). Yeast transformations were performed using the lithium acetate protocol (15).

Spontaneous mutants resistant to 7 mM ortho-phenoxa-vanadate were isolated essentially as described previously (1) using MCY1093 or MCY1094 as the starting strains. Resistant colonies arose at a frequency of about 10^-5 after 3-5 days of incubation at 30°C. Two independent spontaneous vanadate-resistant mutant strains, NDY17 and NDY1.4, were back-crossed twice to the parental strain MCY1093. Tetrad analysis of these back-crossed strains demonstrated that for both mutants, the glycosylation defect, vanadate resistance, and hygromycin resistance cosegregated as a single locus.

Oligosaccharyltransferase Activity Assay—The peptide substrate, containing the recognition sequence for N-linked oligosaccharide addition, was prepared by iodinating Asn-Lys(N-p-azidobenzoyl)-Thr-NH2 with 125I-labeled Bolton-Hunter reagent as described previously (10). Oligosaccharyltransferase activity was assayed in vitro as described previously (16).

Briefly, cells were grown to logarithmic stage, and spheroplasts were prepared by digestion with Zymolyase 100T (ICN Radiochemicals, Irvine, CA), washed and resuspended in glycosylation buffer (50 mM Tris-HCl, pH 7.4, 10 mM MgCl2, 1 mM 2-mercaptoethanol). These lysates were incubated for 20 min with 75 ng of HpaI/Lys(BNzN)-Thr-NH2 to allow glycopeptide formation in vitro. After stopping the reaction by the addition of Nonidet P-40 to 1%, labeled glycopeptide was separated from unglycosylated peptide by binding to concanavalin A-agarose beads (Sigma) and quantitated by gamma scintillation counting.

Immunoblotting and Peptide N-Glycosidase Digestions—Prior to protein extraction, stationary cultures were diluted to an A500 of about 0.5 and grown for 4-5 h. 120 μl of 2.0 mM NaOH, 8% 2-mercaptoethanol was added directly to 0.8 ml of cell cultures (1-2 x 10^7 units). After 10 min at 0°C, 120 μl of 50% trichloroacetic acid was added. After an additional 10 min at 0°C, the mixture was centrifuged in a microcentrifuge for 3 min at room temperature at 14,000 rpm. The protein precipitate was washed vigorously in 1 ml of 50% trichloroacetic acid for at least 1 min and centrifuged, and the pellet was resuspended in 75 μl of sample buffer, containing 60 μl Tris-HCl (pH 6.8); 2% SDS; 10% glycerol; 5% 2-mercaptoethanol; 1 mM phenylmethylsulfonyl fluoride; 0.1% bromphenol blue). Samples (3-5 μl) were fractionated on 9% SDS-polyacrylamide gels.

Analysis of carboxypeptidase Y (CPY) and the yeast binding protein

HMS1 were used as templates for the synthesis of RNA in vitro. Both plasmids contained the OST4 gene in the same orientation as T7 and T3 promoters, which flank the polylinker of pRS316. In vitro transcription reactions, using either T7 or T3 RNA polymerase, were performed according to manufacturer’s instructions (Promega). In vitro translation reactions in rabbit reticulocyte lysates were performed according to the manufacturer’s instructions (Promega), except that reactions were performed in a total volume of 10 μl instead of 50 μl. In vitro translated proteins, radiolabeled with [35S]methionine or [35S]cysteine (DuPont NEN) were fractionated on 15% SDS-polyacrylamide gels (20). Gels were processed by fixation in 10% methanol, 10% acetic acid, followed by fluorography.

**RESULTS**

Identification of Vanadate-resistant Early Glycosylation Mutants—Sodium vanadate is an effective agent for the enrichment of mutants defective in processes that affect glycosylation (1). Spontaneous mutants were isolated on media containing 7-10 mM sodium vanadate. Complementation analyses indicated that 28 mutants obtained in this manner represent a minimum of eight different genes (data not shown). As expected, we isolated alleles of previously described genes that regulate glycosylation, including MNN9, VAN1 (aka VRG7), and VRG4. The MNN9 (21), VAN1 (22), and VRG4 (34) genes have been cloned. Mutations in all of these genes indirectly affect glycosylation processes known to occur in the Golgi complex.

We analyzed the steady state levels of the vacuolar protein, CPY, in all of these mutants by immunoblot analyses using

**TABLE I**

| Strain         | Genotype     | Source                      |
|---------------|--------------|-----------------------------|
| MCY1093       | MATa his4::359 lys2::B01 ura3-52 | M. Carlson, Columbia University |
| MCY1094       | MATa ade2-101 ura3-52            | M. Carlson, Columbia University |
| NDY17        | MATa ade2-101 ura3-52 ot4-2      | This study                   |
| NDY1.4       | MATa ade2-101 ura3-52 ot3-4      | This study                   |
| NDY21       | MATa ade2-101 ura3-52 his4::359 leu2-3,112 ot4-2 | This study                   |
| JCY11        | MATa ade2-101 ura3-1 his3-11 his3-11 trpl1-1 leu2-3,112 can1-100 ot4A::URA3 | This study                   |
| JCY12        | MATa ade2-1 ura3-1 his3-11 trpl1-1 leu2-3,112 cam1-100 6X::URA3 | This study                   |
| W3031a       | MATa ade2-1 ura3-1 his3-11 trpl1-1 leu2-3,112 cam1-100 | R. Rothstein, Columbia University |
| W3031b       | MATa ade2-1 ura3-1 his3-11 trpl1-1 leu2-3,112 cam1-100 | R. Rothstein, Columbia University |
| W3032n       | MATa ade2-1 ura3-1 his3-11 trpl1-1 leu2-3,112 cam1-100 | R. Rothstein, Columbia University |
| m163         | MATa ade1 ade2 ura lys2 tyr1 his7 gal1 ot1-1 | William Lennarz, SUNY |

Samples were applied directly to 9% SDS-polyacrylamide gels, transferred to nitrocellulose, and probed with anti-CPY antisemur.

Cloning and Sequencing the OST4 Gene—The ost4-2 strain, NDY21, was transformed with a CEN-based yeast genomic library (from Phil Hieter). Leu+ transformants were replica plated onto YPAD plates supplemented with 30 μg/ml hygromycin B. This concentration was previously shown to be inhibitory for growth of these mutants (17). Plasmid DNA was isolated from hygromycin B-resistant colonies and introdiced into Escherichia coli by electroporation. These plasmids were then retransformed back into NDY21 to test for complementation and subjected to restriction analysis.

A 1.1-kb EcoRI fragment containing the complementing activity was sequenced by the dideoxy method (18). For one strain, a series of nested deletions was generated using the exonuclease III/S1 nuclease method (19). A series of synthetic oligonucleotide primers were subsequently used to sequence the complementary strand. The 375-bp HindII/Xhol fragment in pg HC3, which contains the sequence required for complementing the ost4 mutant, was subjected to more rigorous sequence analysis. Sequencing reactions, using both dGTP and dTTP labeling mixtures (Sequenase, Version 2.0; U.S. Biochemical) were analyzed by a variety of gel systems, including 0.5–5% TBE (1 x TBE is 90 mM Tris borate, 2 mM EDTA) gradient gels, containing 6% polyacrylamide (5.7: 0.3 acrylamide to bisacrylamide), 7 m urea; Long Ranger gels, as described by the manufacturer (AT Biochem); or standard 6% polyacrylamide gels containing 40% formamide as described in the Sequenase version 2.0 instruction manual (U. S. Biochemical) to resolve reaction products.

In Vitro Transcription and Translation—Plasmids pg HC3 or pg HC6 were used as templates for the synthesis of RNA in vitro. Both plasmids contained the OST4 gene in the same orientation with respect to the T7 and T3 promoters, which flank the polylinker of pRS316. In vitro transcription reactions, using either T7 or T3 RNA polymerase, were performed according to manufacturer’s instructions (Promega). In vitro translation reactions in rabbit reticulocyte lysates were performed according to the manufacturer’s instructions (Promega), except that reactions were performed in a total volume of 10 μl instead of 50 μl. In vitro translated proteins, radiolabeled with [35S]methionine or [35S]cysteine (DuPont NEN) were fractionated on 15% SDS-polyacrylamide gels (20). Gels were processed by fixation in 10% methanol, 10% acetic acid, followed by fluorography.

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We analyzed the steady state levels of the vacuolar protein, CPY, in all of these mutants by immunoblot analyses using
OST4–3

lanes 2

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Fig. 1. Glycosylation states of carboxypeptidase Y in different vanadate-resistant mutants. Proteins were extracted from isogenic wild-type cells (MCY1094) (lane 1) or the vanadate-resistant mutants ost4–2 (NDY17.4) (lane 2), or ost4–3 (NDY1.4) (lane 3) and subjected to immunoblot analysis with antisera against CPY as described in “Materials and Methods.” The mobility (m) of the mature, vacuolar form of CPY is indicated with an arrow.

anti-CPY antiserum. From this analysis, we were surprised to find two mutants that were defective in early steps of the glycosylation pathway that occur in the ER. All previously characterized vanadate-resistant were defective in Golgi-localized glycosylation. Normally, as CPY transits the secretory pathway, it undergoes a series of modifications. These include a glycosylated ER form, containing core oligosaccharides (67 kDa) added at four positions; a Golgi form that is further modified by the addition of sugars (69 kDa); and a proteolytically processed vacuolar form (61 kDa) (23). In wild-type cells at steady state, CPY is predominantly found as the mature, 61-kDa vacuolar form. Two independent vanadate-resistant isolates accumulated forms of CPY that migrated with an increased electrophoretic mobility on SDS-polyacrylamide gels compared with that of the mature, vacuolar form normally found in wild-type cells (Fig. 1, compare lane 1 with lanes 2 and 3). In these two allelic mutants, which we designate ost4–2 and ost4–3 (see below), CPY accumulated as a series of intermediate species, each differing by about 2 kDa (Fig. 1, lanes 2 and 3). This ladder-like pattern of CPY mobility is strikingly similar to that described by Aebl and co-workers (4, 5) in their analyses of the yeast oligosacaryltransferase mutants wpb1 and swp1. OTase catalyzes the earliest step in N-linked glycoprotein addition: the transfer of the core oligosaccharide to an asparagine residue of nascent proteins in the ER. CPY has four sites for N-linked glycosylation. Leaky mutations in OTase result in the underglycosylation of CPY, with the accumulation of discrete intermediates in which zero, one, two, three, or four of these sites are utilized. Intermediates of CPY that accumulate in mutants with defects in the synthesis of the core oligosaccharide migrate as a smear rather than a ladder. The increased mobility of CPY in these ost4 mutants therefore suggested a defect due to a reduction in the number or size of core oligosaccharides that are added in the ER.

To establish that the CPY intermediates that accumulated in ost4–2 and ost4–3 represent underglycosylated CPY rather than proteolytic products, protein extracts were treated with peptide N-glycosidase prior to fractionation by polyacrylamide gel electrophoresis. This enzyme removes N-linked oligosaccharides from proteins. If the mobility shift in CPY observed in the mutants is due solely to underglycosylation, then enzymatic deglycosylation after peptide N-glycosidase digestion should result in a single nonglycosylated form of CPY that migrates identically in both mutant and wild-type cells. As shown in Fig. 2, this was the case. A time course of peptide N-glycosidase-catalyzed deglycosylation of CPY in extracts from wild-type cells resulted in the progressive cleavage of one, two, three, or four oligosaccharides (Fig. 2, lanes 2–5). These underglycosylated forms of CPY precisely comigrated with those seen in ost4–2 extracts that had not been treated with peptide N-glycosidase (Fig. 2, lane 6, labeled ost4). After 6 h of peptide N-glycosidase digestion, CPY from both ost4–2 and ost4–3 (data not shown) accumulates almost exclusively as a fully deglycosylated form that is identical to the deglycosylated form seen in wild-type cells (Fig. 2, compare lanes 5 and 7). This result demonstrates that the aberrant CPY intermediates that accumulate in ost4–2 and ost4–3 strains are due to underglycosylation rather than proteolysis of the mature CPY protein.

Fig. 2. Peptide N-glycosidase digestion of carboxypeptidase Y in OST4 and ost4–2 mutant extracts. Total cellular proteins were extracted from OST4 (strain MCY1094) (lanes 1–5) or ost4–2 cells (strain NDY17.4) (lanes 6 and 7) and treated with buffer alone (lanes 1 and 6) or digested with buffer containing peptide N-glycosidase for the times indicated (in hours) above each lane. Proteins were then fractionated on SDS-polyacrylamide gels and subjected to immunoblot analysis with antisera against CPY as described under “Materials and Methods.” The mobility of the fully glycosylated vacuolar form of CPY (CPYm) is indicated with an arrow.

2 N. Dean, unpublished data.

2 ost4–2 and ost4–3 Are Allelic to a Previously Identified OTase Mutant—To determine if ost4–2 is allelic to any previously identified OTase mutants, complementation analyses
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Table II

| Strain    | Relevant genotype | In vitro activity |
|-----------|-------------------|------------------|
| MCY1094   | OST4              | 100              |
| m163      | ost4–1            | 9                |
| NDY17.4   | ost4–2            | 5                |
| NDY1.4    | ost4–3            | 7                |

with mutants in a number of different genes that affect OTase activity were performed. We have previously shown that most early glycosylation mutants, including ost4–2 and other OTase mutants, are sensitive to growth on the aminoglycoside, hygromycin B (17). Therefore, complementation of the mutant phenotype was assayed by the ability of cells to grow on medium supplemented with 30 μg/ml hygromycin B. Transformation of the ost4–2 strain, NDY17.4 with plasmids containing the WBP1, or SWP1 genes failed to rescue the mutant phenotype, suggesting that neither of these genes encoded the wild-type function defective in NDY17.4.

Genetic crosses between the OTase mutant, m163 (19), and either ost4–2 or ost4–3 resulted in diploid strains that failed to grow on medium supplemented with hygromycin B, suggesting that ost4–2 and ost4–3 are allelic to the OTase mutant previously identified by Roos et al. (10). Because this allele was previously mentioned in the literature (10) but had not been given a name, we have designated m163 as ost4–1. Tetrads analysis of 12 sporulated diploids produced by crossing ost4–1 with either ost4–2 or ost4–3 exhibited complete linkage of hygromycin B sensitivity and confirmed that these mutants are indeed allelic (data not shown). Extracts prepared from strain m163 indicate that ost4–1 is defective in peptide glycosylation activity in vitro (Ref. 10 and see Table I). ost4–1 is also vanadate-resistant and hygromycin B-sensitive. Unlike ost4–2 and ost4–3, ost4–1 is temperature-sensitive for growth and even at the permissive temperature, grows much more slowly than ost4–2 or ost4–3. Gel filtration analysis of purified oligosaccharide isolated from the oligosaccharidyl pyrophosphoryl dolichol synthesized in vivo demonstrated that a full-length core is synthesized by m163, indicating that the ost4–1 mutation does not substantially affect the synthesis of the core oligosaccharide (10). While this assay is not sufficiently sensitive to detect a loss of one or two sugars, these results strongly suggest that the primary defect in ost4–2 and ost4–3 is in the transfer of oligosaccharide. We propose to name the wild-type gene defined by these mutants OST4 (gligo saccharide transferase 4).

Cloning of the OST4 Gene—The OST4 gene was cloned by transforming NDK2I, containing the ost4–2 allele with a CEN4-based yeast genomic library containing the LEU2 selectable marker (from P. Hieter). Leucine prototrophic transforms were replica-plated on YPAD containing 30 μg/ml hygromycin B, and drug-resistant colonies were isolated. Plasmid DNA from these hygromycin B-resistant colonies was isolated and retested for complementation of the mutant phenotype (see Fig. 3). Four different plasmids were identified by this assay, each containing related overlapping restriction fragments. Each of these plasmids also rescued the mutant glycosylation defect, assayed by immunoblots using CPY antiserum (data not shown). Independently, the ost4–1 was transformed with the same yeast genomic library, and a temperature-resistant transformant was isolated. Plasmid DNA was recovered, and restriction analysis indicated that it contained overlapping restriction fragments with the plasmids that rescued mutant ost4–2. Upon further subcloning and complementation analy-

Figure 3. Complementation of hygromycin B sensitivity of the ost4–2 mutation by pOST4. Isogenic OST4 (MCY1094) and ost4–2 mutant strains (NDY17–4), harboring either control parental plasmid, or a plasmid containing the 1.1-kb EcoRI complementing fragment were streaked onto either YPAD (lower panel) or YPAD plates supplemented with 30 μg/ml hygromycin B (upper panel).
the ost4 mutation led to the suspicion that this small ORF was prematurely truncated due to a sequencing error. Alternatively, we considered the possibilities that either the 3' untranslated region absent in the HindIII/ScaI fragment was somehow required for RNA expression or stability, or that this DNA does not encode a protein.

Upon examination of the predicted open reading frame map (Fig. 5C), we noted that two reading frames (underlined in Fig. 5A) could be joined by the addition of a single base, resulting in an ORF of 276 bp that could encode a 10-kDa protein. As the purified yeast OTase complex includes a polypeptide of about 9 kDa (8), it was assumed that a sequencing error had occurred, despite the clarity in this region. The sequence in the area surrounding the putative frameshift region, AAGAAC, does not conform to GC-rich regions normally associated with artificial compressions (see Fig. 5A). Moreover, the sequence in this region was unambiguous with respect to both the resolution and spacing of bases on both strands (data not shown). After exhaustive sequencing efforts, in which a number of different gel and reaction conditions were used, no sequencing errors in this region could be identified (data not shown).

Given the small size of the complementing fragment, we first wished to confirm that this DNA encoded a protein-encoding RNA. If this were the case, the introduction of a simple frameshift mutation in the ORF, by insertion or deletion of one or two bases, should result in loss of function. Alternatively, if this DNA encoded an untranslated RNA, a frameshift mutation might have a minimal effect. To test this idea, we took advantage of a unique StyI restriction site at the 5'-end of the putative open reading frame. Digestion with StyI results in 5'-overhangs of four bases. After digestion with StyI, the ends were filled in with Klenow, thus introducing four additional base pairs (at the StyI site) in this fragment, introducing a frameshift.
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Fig. 6. In vitro translation of Ost4p in the presence of either [35S]cysteine or [35S]methionine. pJHC3, containing the HindIII/Xhol fragment was used as template for the synthesis of OST4 RNA in vitro. In vitro translation reactions containing either [35S]methionine (lanes 1 and 3) or [35S]cysteine (lanes 2 and 4) were performed. Lanes 1 and 2 contain protein derived from translation of a control RNA that encodes a protein that containing both methionines and cysteines residues, demonstrating the fidelity of [35S]cysteine-containing reactions. Lanes 3 and 4 contain protein derived from OST4 RNA.

A search of nucleotide sequence data bases using the BLAST algorithm (26) provided further evidence to support the notion that this small gene encodes a protein. The S. cerevisiae OST4 cDNA sequence was identified in the Expressed Sequence Tags data base (dbest, accession number T36335). This data base contains cDNA sequences derived from mRNA. A comparison of the two OST4 sequences demonstrated these to be identical where the overlap occurred, except for two ambiguities (N) in the deposited sequence and an inversion of two bases. None of these changes could result in the formation of another or extended ORF. The deposited cDNA sequence extended from nucleotide position 85 (as shown in Fig. 5) to approximately 80 bases 3' of the Xhol site.

The OST4 Gene Directs the Synthesis of a 3.6-kDa Protein in Vitro—To determine if the 375-base pair HindIII/Xhol fragment contains the information to direct synthesis of a protein molecule, RNA transcribed by T7 polymerase from plasmid pJHC3 was tested for the in vitro synthesis of [35S]methionine-labeled protein in rabbit reticulocyte lysates. The results of this experiment demonstrated that a small protein, migrating with a molecular weight of 3.6-kDa was translated in vitro (data not shown). The predicted OST4 sequence predicts that the encoded protein is very hydrophobic (Fig. 5B). Since the mobility of hydrophobic proteins on denaturing SDS-polyacrylamide gels can lead to an inaccurate estimation of molecular weight, we still considered the possibility that Ost4p may be not encoded by the small 108-base pair ORF but rather by the larger potential ORF generated if one considers the possibility of an artificial frameshift. To distinguish between these two possibilities, we took advantage of the fact that the smaller 108-bp ORF does not contain any cysteines, while the second larger ORF contains two cysteines (see Fig. 5A). Therefore, which of these two proteins is translated could be ascertained simply by assaying the incorporation of [35S]cysteine in vitro. If the small 108-base pair ORF encodes Ost4p, no [35S]cysteine should be incorporated in vitro. The result of this experiment, shown in Fig. 6, demonstrated that this was the case. RNA transcribed in vitro was used to direct translation in rabbit reticulocyte lysates, in the presence of either [35S]methionine or [35S]cysteine (labeled Met and Cys in Fig. 6). An unrelated RNA, coding for a 52-kDa zinc finger protein containing 10 methionines and 17 cysteine residues was used to control for the fidelity of the [35S]cysteine-containing reactions (Fig. 6, lanes 1 and 2, labeled control). In translation reactions directed by OST4 RNA, labeled protein was only detected in those reactions containing [35S]methionine (Fig. 6, lane 3), but not [35S]cysteine (Fig. 6, lane 4). From this experiment, we conclude that the small 108-bp ORF encodes the Ost4 protein, which migrates with an apparent molecular mass of 3.6 kDa.

The OST4 Gene Is Essential for Growth at 37°C—We analyzed the effect of deleting the wild-type OST4 gene by replacing it with the URA3 gene (Fig. 7B). A standard one-step gene disruption (27) was performed to replace the chromosomal copy of the OST4 gene (Fig. 7A) with the null allele in a diploid. The disruption of one allele was confirmed by Southern blot analysis of genomic DNA (data not shown).

Heterozygous diploids were sporulated, and dissected tetrads were analyzed for cell viability at 25°C (Fig. 7C). This experiment demonstrated that the OST4 gene encodes a protein that is not essential for viability at 25°C but that is important for normal growth. Colonies from spores carrying the null allele (i.e. Ura') could not be detected until 5 days after the wild-type colonies arose (Fig. 7C). Cells carrying the null mutation grew more slowly than those carrying either the ost4-1 or ost4-2 alleles. The growth of four spores from a single tetrad was also compared at 25 and 37°C. No growth was observed in ost4::URA3-disrupted colonies grown at 37°C (Fig. 7D), even on plates containing osmotic stabilizers (data not shown). We therefore conclude that the OST4 gene is necessary for normal growth at room temperature and is essential for growth at high temperatures.

In S. cerevisiae genes encoding four components of OTase have been identified: WBP1 (4), SWP1 (5), OST1 (6), and OST3 (7). WBP1, SWP1, and OST1 encode proteins that are essential for viability. As OST4 is only required for growth at higher temperatures, we wished to determine what effect the loss of OST4 function had on OTase activity at the nonrestrictive temperature of 30°C. Protein extracts were prepared from the disruption strains derived from each of the four sister spores shown in Fig. 7 and subjected to immunoblot analysis to detect any changes in the level of CPY glycosylation (Fig. 8A). This experiment demonstrated that the loss of OST4 function results in a marked decrease in OTase activity in vivo. In this experiment, the ratio of underglycosylated CPY to fully glycosylated CPY was increased in cells containing the null allele compared with the mutant, ost4–2 (Fig. 8A, compare lanes 4 and 5 with lane 6). We have observed some experiment variability in the ratio of the different intermediates that accumulate in all of the ost4 mutant strains (compare, for example, Fig. 1, lane 2, and Fig. 8A, lane 6). This may result from the growth stage at which cells are harvested, which may in turn affect the rate of CPY transport from the ER, allowing more time with which oligosaccharides may be transferred. In the absence of OST4 gene product, there remained a low, but detectable, amount of fully glycosylated CPY that comigrated with the mature form seen in wild-type cells (Fig. 8, compare lanes 4 and 5 with lane 1).

The unglycosylated precursor form of CPY migrates with a molecular mass of 59 kDa, similar to that of the fully glycos-
OST4 Is Required for OTase Activity

Fig. 7. The OST4 gene is essential for growth at 37 °C. Panel A is a schematic representation of the restriction map of the region surrounding the OST4 gene. Panel B depicts the strategy used to create the ost4:URA3 disruption plasmid. Panel C, shows tetrath analysis of diploid strains heterozygous for the ost4-disrupted allele. Tetrads obtained from the sporulation of strain JCY11 were dissected on YPAD plates. The spores were incubated for 5 days at 30 °C. Each column is labeled numerically, and each spore is labeled alphabetically. Two large spores and two small spores from each tetrad are evident. All large spores were Ura+ and all small spores were Ura-. Panel D, columns from four spores of one tetrad obtained from the diploid strain heterozygous for the ost4 deletion (shown on panel C) were streaked onto YPAD plates and incubated at 25 or 37 °C for 4 days.

Fig. 8. Glycosylation states of CPY in wild-type, ost4-2, and the ost4::URA3 mutant. Protein was extracted from yeast and subjected to immunoblot analysis using antisera against carboxypeptidase Y, as described under “Materials and Methods.” Panel A, proteins were prepared from OST4 cells (MCY1094) (lane 1), NDY17.4, containing the ost4-2 allele (lane 6), or from strains derived from the four spores of one tetrad derived from the ost4::URA3 diploid, J CY11 (see Fig. 7C) containing the wild-type OST4 allele (lanes 2 and 3) or a deletion of the OST4 gene (lanes 4 and 5). The position of the mature vacular form of CPY is indicated with an arrow; the position of the underglycosylated forms are indicated by the bracket. Panel B, proteins were extracted from OST4 cells (MCY1094) (lanes 3 and 4), NDY17.4, containing the ost4-2 allele (lanes 5 and 6), or J CY12, containing ost4::URA3 null allele (lanes 1 and 2) and treated with buffer alone (lanes 1, 3, and 5) or digested with buffer containing peptide N-glycosidase for 8 h (lanes 2, 4, and 6). Proteins were then fractionated on SDS-polyacrylamide gels and subjected to immunoblot analysis with antiserum against CPY as described under “Materials and Methods.”

A large body of data suggests that correct glycosylation is required for the proper folding of many glycoproteins (28). Therefore, it was of interest to ascertain whether or not the temperature-sensitive lethality of the ost4 null mutant may reflect a temperature-dependent protein folding requirement for glycosylation. To test this, we assayed the level of Kar2p (BiP), whose synthesis is normally induced in response to unfolded proteins in mutant and wild-type cells grown at 25 or 37 °C (7). Sogenic wild-type and ost4::URA3 null mutants were grown to mid log phase at 25 °C, after which aliquots of cells were removed and shifted to growth at 37 °C. After 12 additional h of growth, equal numbers of cells were centrifuged, and both the intracellular and extracellular levels of Kar2p was assayed by immunoblot analysis. In wild-type cells, the amount of intracellular Kar2p increased by 5-fold at 37 °C compared with 25 °C (Fig. 9, compare lanes 1 and 3), and none was secreted at either temperature (Fig. 9, lanes 2 and 4). This was expected since the synthesis of Kar2p, a member of the heat shock family of proteins, is known to be induced as a result of high temperature. Even after induction, its level was apparently below that required to saturate the normal ER retention system, and it was not secreted into the culture supernatant. In the ost4 null mutant, at 25 °C, Kar2p was at levels similar to those seen in wild-type cells grown at 37 °C, suggesting that the protein unfolding response was induced 5-fold even at the permissive temperature. Kar2p synthesis did not significantly increase when ost4::URA3 cells were grown at 37 °C (Fig. 9, compare lanes 5 and 7). There was, at most, a 1.5-fold increase. No Kar2p was secreted into the culture media at either temperature (Fig. 9, lanes 6 and 8). These results suggest that this mutation leads to an accumulation of misfolded proteins. The lack of further induction at high temperature in the null mutant may reflect the possibility that Kar2p induction was already at a maximal levels at the permissive temperature.
We have described the isolation of vanadate-resistant mutants that are defective in early steps of N-linked glycosylation. We demonstrate that the ost4–2 and ost4–3 mutants are defective in OTase activity both in vivo and in vitro and are allelic to a previously identified OTase mutant, m163 (designated ost4–1). In addition to the two isolates carrying the ost4 mutation, we also identified another, as yet uncharacterized vanadate-resistant mutant with defects in early glycosylation (data not shown). While the basis for resistance to sodium vanadate is unknown, it has been demonstrated to be a useful agent for the enrichment of mutants defective in steps that affect glycosylation in the Golgi complex (1). The isolation of early glycosylation mutants described here suggests that it may be useful for the selection of early glycosylation mutants as well.

The nucleotide sequence of the DNA fragment that complements the ost4 mutant identified a small ORF, predicted to encode a 3.9-kDa protein. While many proteins of this size exist, most are proteolytic products of preproteins. The small, almost unprecedented, predicted size of this protein led us to initially suspect that an error had occurred in determining the DNA sequence, despite rigorous sequencing efforts. Also, complementation analysis with pJHC4, which contains a fragment of the DNA sequence, despite rigorous sequencing efforts. Also, complementation analysis with pJHC4, which contains a fragment of the DNA sequence, suggested that the small protein is a component of the observed yeast protein complex (data not shown). While the basis for resistance to sodium vanadate is unknown, it has been demonstrated to be a useful agent for the enrichment of mutants defective in steps that affect glycosylation in the Golgi complex (1). The isolation of early glycosylation mutants described here suggests that it may be useful for the selection of early glycosylation mutants as well.

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We first demonstrated that this gene encodes a protein, rather than an untranslated RNA. An insertion mutation, introduced just downstream the initiating methionine resulted in the loss of OTase function. Furthermore, treatment of microsomes with RNase had no effect on oligosaccharyltransferase activity in vitro (data not shown). A search of nucleotide sequence data bases provided further evidence to support the notion that this small gene encodes a protein. The OST4 sequence was identified in the dbEST data base, which contains cDNA sequences derived from mRNA. Finally, this DNA contains the information for the synthesis of protein in vitro. Differential labeling with [35S]cysteine versus [35S]methionine clearly demonstrated that the in vitro translation product is a protein that migrates with a molecular mass of 3.6 kDa, containing no cysteine residues. If one invoked a frameshift in the DNA sequence, the 10-kDa translation product would contain two cysteines. These results, taken together, demonstrate that the OST4 gene encodes an unusual protein, merely 36 amino acids in length, predicted by hydropathy analysis to lie almost entirely in a membrane.

The complete absence of the OST4 gene product resulted in a severely compromised growth rate at normal temperature and temperature-sensitive lethality. One explanation for such a phenomenon is that lethality is an indirect effect of the glycosylation defect that is manifested only at high temperatures. An obvious candidate for such a temperature-sensitive process is protein folding. Oligosaccharyltransferase addition is necessary for the proper folding or assembly of many glycoproteins (28). A number of glycosylation mutants exhibit temperature-sensitive growth phenotypes, both in yeast (2, 29, 30) and mammalian cell lines (31); and inhibition of glycosylation is known to affect the folding of many proteins (28, 32). At the higher temperature, the folding of some glycoprotein(s) required for viability may be impaired in ost4 mutants. We tested this by monitoring the synthesis of Kar2p, whose induction occurs in response to misfolded proteins (7). We found high levels of Kar2p induction in the null mutant even at the permissive temperature, suggesting a defect in protein folding.

An alternative explanation is that Ost4p facilitates or maintains the assembly of the OTase polypeptide complex, whose activity is required for viability. In the absence of Ost4p at the permissive temperature, complex formation may be impaired but can be tolerated. At higher temperatures, the complex is unstable, resulting in an inactive enzyme. Consistent with this notion is the correlation between the severity of the glycosylation defect and the growth defect observed in ost4 mutants.

What is the function of Ost4p? A 3.6-kDa protein was not observed as a component of the purified yeast protein complex (8, 9). However, the identification of polypeptides in this size range would not be expected by conventional SDS-polyacrylamide gel electrophoresis. Consequently, it is quite possible that Ost4p is a structural component of oligosaccharyltransferase. Ost4p is predicted to be a very hydrophobic membrane protein, containing a single membrane-spanning domain. Indeed, little of the protein sequence is predicted to be anywhere but buried in a membrane, raising questions concerning what portion of the protein may be available for interaction with other proteins outside of the membrane. No dolichol-binding consensus sequence is obvious in the membrane-spanning portion of Ost4p. Experiments designed to map the mutations in each of the different ost4 alleles may help to address this issue. Based on its predicted membrane localization, it seems likely that this protein is a structural component of OTase, or a proximal accessory component that regulates OTase activity or assembly. The observation that mature, fully glycosylated CPY accumulates in the complete absence of Ost4p suggests that it is not an essential component. Despite this, the dramatic effect that the loss of OST4 function has on the normal growth rate of cells and on the efficiency of glycosylation demonstrates its importance for normal OTase activity. The identification of OST4 and the other genes that encode subunits or regulators of OTase will enable us to define how this multifunctional enzyme functions in glycosylation.

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