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A Deletion That Includes the Signal Peptidase Cleavage Site Impairs Processing, Glycosylation, and Secretion of Cell Surface Yeast Acid Phosphatase

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We transformed *Saccharomyces cerevisiae* with a high-copy-number plasmid carrying either the wild-type gene coding for a repressible cell surface acid phosphatase or two modified genes whose products lack a 13- or 14-amino-acid segment spanning or immediately adjacent to the signal peptidase cleavage site. The wild-type gene product underwent proteolytic cleavage of the signal peptide, core glycosylation, and outer chain glycosylation. The deletion spanning the signal peptidase cleavage site led to an unprocessed protein. This modified protein exhibited core glycosylation, whereas its outer chain glycosylation was severely inhibited. Secretion of the deleted protein was impaired, and active enzyme accumulated within the cell. The deletion immediately adjacent to the signal peptidase cleavage site exhibited only a small decrease in the efficiency of processing and had no effect on the efficiency of secretion.

Studies on the biosynthesis of secretory proteins in procaryotes and eucaryotes have revealed that most of the proteins are synthesized in vitro as precursors with 15 to 30 additional amino acids at the NH_2-terminal end of the molecule (31). This transient NH_2-terminal extension has been termed the signal peptide (6). It has been shown in eucaryotes that upon emergence of the signal peptide from the large ribosomal subunit, the ribosomal complex makes contact with the membrane of the endoplasmic reticulum (ER) by binding to the signal recognition particle (48), and the resulting complex binds in turn to the docking protein (28). The nascent protein is then cotranslationally translocated across the membrane of the ER, and the signal peptide is cleaved by signal peptidase (22).

In eucaryotes, protein secretion is often linked to protein glycosylation within the ER and the Golgi apparatus. The pathway of glycoprotein secretion in yeasts is similar to that described for higher eucaryotes (35), with only slight modifications. Isolation and characterization of numerous temperature-sensitive yeast secretory mutants blocked at various stages of this pathway (32, 33) led to the following model (15). The initial N-linked asparagine core glycosylation of secretory proteins probably occurs during translocation of the polypeptide through the membrane of the ER. Core oligosaccharides are then extended in a Golgi-like organelle by the addition of outer chain sugar branches (3). Fully glycosylated proteins are then packed into vesicles which are transported to the bud, here they fuse with the plasma membrane and discharge their contents into the periplasmic space (32). This model should apply to repressible acid phosphatase. Although this enzyme has been studied extensively, it is not yet clear whether its final localization is in the periplasm (2) or in the cell wall (4, 25). Moreover, some of the enzyme is excreted (8).

Although genetic approaches have significantly contributed to our understanding of the essential features of signal peptides in protein transport in *Escherichia coli* (17, 30), similar studies have only recently been reported for eucaryotic systems (16, 42). We have previously isolated and characterized two yeast acid phosphatase structural genes (*PHO5* and *PHO3*) arranged in tandem (29), both of which code for proteins secreted to the surface of the cell. The expression of the *PHO5* gene is repressed at high levels of phosphate, whereas the gene *PHO3* is constitutively expressed. Our initial sequencing data of the *PHO5* gene revealed a stretch of 20 neutral amino acids at the amino terminus of the protein, which strongly suggested the presence of a signal peptide. Amino acid sequence analysis of the mature protein (1, 46; unpublished data) was consistent with the existence of a signal peptide consisting of 17 amino acids. The presence of unique restriction sites flanking the presumed processing site (*BalI*, *SalI*, and two *KpnI* sites; see Fig. 1) enabled us to construct small deletions on either side of the processing site. We present here the results of an analysis of two deletions of *PHO5* [Δ*BalI-SalI*(ΔBS) and Δ*KpnI*(ΔK)] beginning, respectively, before and immediately after the signal peptidase cleavage site. We show that processing, glycosylation, and secretion of regulated acid phosphatase are affected in the ΔBS mutant, whereas processing and secretion are normal in the ΔK mutant. These results have been presented in part previously (19).

MATERIALS AND METHODS

Bacteria strains. *E. coli* HB101 (*hsdR hsdM leu pro recA*) was used for plasmid preparations. JM101 (Δ*lac pro supE thi* F′ *traD36 proAB lacI*^{q}ZΔM15) was used for M13 cloning.

Yeast strain. *Saccharomyces cerevisiae* GRF18 (α *his3-11 his3-15 leu2-3 leu2-112 can*^{r}) was used.

Bacterial vectors. The phage M13mp8 was constructed by J. Messing (27).

Hybrid bacteria-yeast vectors. p29 is a pBR322 derivative; the DNA segment between *Bam*HI and *Pvu*II was replaced by a 3.9-kilobase (kb) *Bam*HI-*Hpa*I fragment containing *PHO5* and *PHO3* (W. Bajwa, B. Meyhack, H. Rudolph, A. M. Schweingruber, and A. Hinnen, submitted for publica-

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tion). The self-replicating vector pJDB207 was constructed by Beggs (5).

Bacteria media. HB101 was grown in LB medium (29). JM101 was grown in YT medium containing (per liter) tryptone (8 g), yeast extract (5 g), and NaCl (2.5 g).

Yeast media. YPD medium contained 2% Bacto-Peptone (Difco Laboratories, Detroit, Mich.), 1% yeast extract (Difco), and 2% glucose. Liquid minimal medium was based on Difco yeast nitrogen base without amino acids, to which 2% glucose and the required amino acids (20 mg/liter) were added. Minimal medium plates and slants were made from the same medium, to which 2% agar was added. Low-P_i and high-P_i media were prepared according to the recipes of the above Difco medium with 2 g of asparagine per liter instead of $(NH_4)_2SO_4$ and with 1 g of KH_2PO_4 per liter (high P_i) and 0.03 g of KH_2PO_4 per liter plus 1 g of KCl per liter (low P_i). In the labeling experiments, the $MgSO_4 \cdot 7H_2O$ content of low-P_i medium (0.5 g/liter) has been replaced by $MgCl_2 \cdot 6H_2O$ (0.4 g/liter) plus $MgSO_4 \cdot 7H_2O$ (0.005 g/liter) (low inorganic sulfate S_i medium).

Construction of recombinant plasmids. T4 DNA ligase and restriction endonucleases except *Eco*RI (Boehringer Mannheim Biochemicals, Indianapolis, Ind.) were from New England Biolabs, Beverly, Mass. These enzymes were used as described in the specifications of the suppliers. Restriction fragments were prepared by electrophoresis in low-melting agarose (type Sigma VII). After excising the fragments from the gel, agarose was melted at 65°C, and gel concentration was diluted to 0.3%. Ligation was then performed after mixing appropriate amounts of the required restriction fragments. This mixture was directly used to transform Ca^{2+}-treated (26) HB101 cells or to transfect JM101 cells. Plasmid isolation was as described by Clewell (12).

Yeast transformation. Yeast strain GRF18 grown in YPD medium was transformed as described by Hinnen et al. (18). Samples of the spheroplasts were suspended in 5 ml of regeneration agar, plated on Leu^- yeast minimal medium plates, and incubated for 4 days. Leu^+ transformants were then picked and stored on Leu^- minimal medium slants.

Cell growth and derepression of acid phosphatase. GRF18 as well as transformants were grown overnight in minimal medium plus histidine (20 mg/liter) and leucine (20 mg/liter) (for the parent strain) or histidine alone (for the transformants). They were harvested and suspended at an optical density at 600 nm (OD_{600}) of 0.05 (except in the case of Fig. 3 [OD_{600} of 1]) in low-P_i or high-P_i medium (or low-P_i, low-S_i medium for the in vivo labeling experiments) and further grown overnight. Growth was stopped during mid-logarithmic phase (usually at an OD_{600} of 1.5).

RNA preparation. Total cellular RNA from yeast strains was prepared as described by Meyhack et al. (29) from yeast strains grown and derepressed in low-P_i medium.

Toluene treatment. Toluene treatment was performed as described by Serrano et al. (43), with slight modifications: 50 μl of ethanol-toluene (4:1) was added to 1 ml of yeast cells in growth medium and then submitted to 5 min of vortex agitation (Multivortex; speed 6). Control experiments showed that the efficiency of permeabilization was independent of the amount of cells per milliliter.

Acid phosphatase activity. Intact whole cells in growth medium, toluene-treated cells in growth medium, or supernatant growth medium was assayed for acid phosphatase activity as described by Toh-e et al. (47), with slight modifications. The reaction mixture (0.5 ml) contained 0.05 M acetate buffer (pH 4), *p*-nitrophenylphosphate (0.45 mg/ml), and a suitable amount of enzyme (50 to 100 μl of untransformed cells or supernatant medium and 2 to 10 μl of derepressed transformed cells). The reaction was carried out for 10 min at 37°C and stopped by the addition of 0.12 ml of 25% trichloroacetic acid, followed by 0.6 ml of saturated Na_2CO_3. When necessary, cells were removed by centrifugation before reading the absorbance at 405 nm. One unit of acid phosphatase activity is defined as the amount of enzyme which catalyzes the liberation of 1 μmol of *p*-nitrophenol per min at 37°C.

Cell-free translation. Total yeast RNA (50 μg) was translated at 29°C in a nuclease-treated cell-free reticulocyte system (36) in a total volume of 50 μl in the presence of 100 μCi of [^{35}S]methionine (New England Nuclear Corp., Boston, Mass.), in the presence or absence of purified bacterial leader peptidase (40 μg/ml), and in the presence of 0.12% Triton X-100 which helps the action of leader peptidase. After 45 min of incubation, 1 mM phenylmethylsulfonyl fluoride was added, and after 5 min, the samples were dissociated with 4% sodium dodecyl sulfate for 4 min at 95°C. The dissociation mixture was cooled to room temperature, diluted with 2.5 ml of Triton buffer (50 mM Tris-hydrochloride (pH 8), 140 mM NaCl, 5 mM EDTA, 1% Triton X-100), and further used for immunoprecipitation.

Labeling of yeast cells. Yeast cells grown and derepressed in low-P_i, low-S_i medium were harvested at an OD_{600} of 1.5 and suspended at an OD_{600} of 15 in 40 mM sodium citrate (pH 6) supplemented with 2% glucose and histidine (20 μg/ml). When indicated, tunicamycin (Calbiochem-Behring, La Jolla, Calif.) treatment was then performed by 30 min of incubation at 30°C in the presence of 10 μg of tunicamycin per ml with gentle shaking. Labeling was performed by the addition of 500 μCi of [^{35}S]methionine per ml and by incubation at 30°C with gentle shaking. For subsequent chases, unlabeled L-methionine was added at a final concentration of 20 mM, and the incubation was continued. The effectiveness of each chase in stopping further incorporation into proteins was checked as described by Reid et al. (38). Incorporation was stopped and cells were denatured by the addition of 800 μl of 20% trichloroacetic acid to 200-μl samples. Cells were immediately broken by vortexing them with an equal volume of glass beads (diameter, 0.5 mm) on a

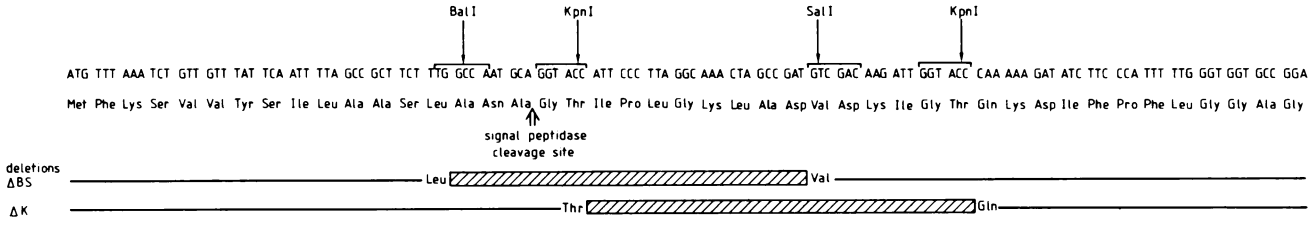

FIG. 1. Nucleotide and amino acid sequences at the beginning of structural genes of *PHO5* and its two deletions derivatives, ΔBS and ΔK.

Vortex mixer for 2 min at maximal speed as described by Ohashi et al. (34). Preparation of the samples for immunoprecipitation was then performed as described by the same authors, with slight modifications. Denatured cells and glass beads were centrifuged, and supernatant was removed. The sedimented proteins were solubilized by adding 1 ml of twofold-concentrated sample buffer (4% sodium dodecyl sulfate, 0.1 M Tris-hydrochloride (pH 6.8), 4 mM EDTA, 20% glycerol, 2% mercaptoethanol, 0.02% bromophenol blue). The mixture was neutralized with 1 M Tris base and heated for 4 min in a boiling bath. The suspension containing denatured solubilized proteins was then carefully removed from the glass beads. The glass beads were rinsed several times with Triton buffer. The rinses were combined with the first mixture and adjusted to a final volume of 20 ml (final sodium dodecyl sulfate concentration, 0.2%). For immunoprecipitation of the supernatant medium, 200-μl samples of labeled cells were centrifuged (2 min; Eppendorf centrifuge), and the proteins of the supernatant medium were precipitated by the addition of 800 μl of 20% trichloroacetic acid and 20 μl of bovine serum albumin (20 mg/ml). The resulting pellet was resuspended in 0.5 ml of twofold-concentrated sample buffer, neutralized by 1 M Tris base, heated in a boiling bath for 4 min, and adjusted to a final volume of 10 ml of Triton buffer.

Immunoprecipitation. All Triton buffer samples resulting from cell-free translation or in vivo labeling were incubated for 30 min at room temperature with 100 μl of a 10% suspension of heat-killed and glutaraldehyde-fixed *Staphylococcus aureus* cells and centrifuged for 20 min at 20,000 × *g*. This step removed insoluble material and labeled proteins binding nonspecifically to bacterial cells. The pellet was discarded. Antiserum specific against purified acid phosphatase was added to the supernatants (10 μl in the case of in vitro samples and supernatant medium samples; 20 μl in the case of total cells), and overnight incubation was performed at room temperature. Fixed *S. aureus* cells (10-fold the volume of antiserum) were added, and the suspension was shaken for 1 h at room temperature. The *S. aureus* cells were then centrifuged, washed four times with 1 ml of Triton buffer, and extracted for 3 min at 95°C with 150 μl of sample buffer. *S. aureus* cells were removed by centrifugation, and the supernatant was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis in 10% polyacrylamide. Gel electrophoresis was performed as described by Douglas and Butow (14), and the dried gels were analyzed by fluorography as described by Chamberlain (11).

RESULTS

Construction of high-copy-number plasmids carrying the *PHO5* gene with a modified signal sequence. Small deletions in the DNA sequence coding for amino acids of the signal sequence, the adjacent mature *PHO5* protein sequence, or both were used to analyze the involvement of this region in secretion and glycosylation. Figure 1 presents the nucleotide sequence at the 5′ end of the *PHO5* coding region (29) as well as the derived amino acid sequence. The amino acids at the NH_2 terminus show features characteristic of a signal sequence: there is a charged amino acid in position 3, followed by 14 neutral amino acids. The putative signal peptidase cleavage site is between amino acids Ala_{17} and Gly_{18} (1, 46). Two deletions have been constructed in plasmid p29 which carries the structural genes for *PHO5* and *PHO3* on a *Bam*HI-*Hpa*I fragment (see Fig. 2) with unique restriction sites (see Fig. 1). Deletion ΔK was obtained by digesting plasmid p29 with restriction endonuclease *Kpn*I and religating the large DNA fragment. The new plasmid p29/*PHO5*/ΔK is characterized by a deletion of 42 base pairs (14 amino acids). Deletion ΔBS was obtained by digesting plasmid p29 with restriction endonucleases *Bal*I and *Sal*I, the 3′-recessed ends of the *Sal*I site were filled in with Klenow DNA polymerase, and the resulting blunt ends of the large DNA fragment were ligated with T4 DNA ligase. This construction recreates a *Sal*I restriction site. The new plasmid (p29/*PHO5*/ΔBS) has a deletion of 39 base pairs (13 amino acids). The positions of the ΔK and ΔBS deletions are shown in Fig. 1. The ΔBS deletion removes three amino acids of the signal sequence, including the signal peptidase cleavage site and amio acids from the mature *PHO5* amino acid sequence. The ΔK deletion leaves the signal sequence and the signal peptidase cleavage site intact but removes 14 amino acids from the mature *PHO5* protein.

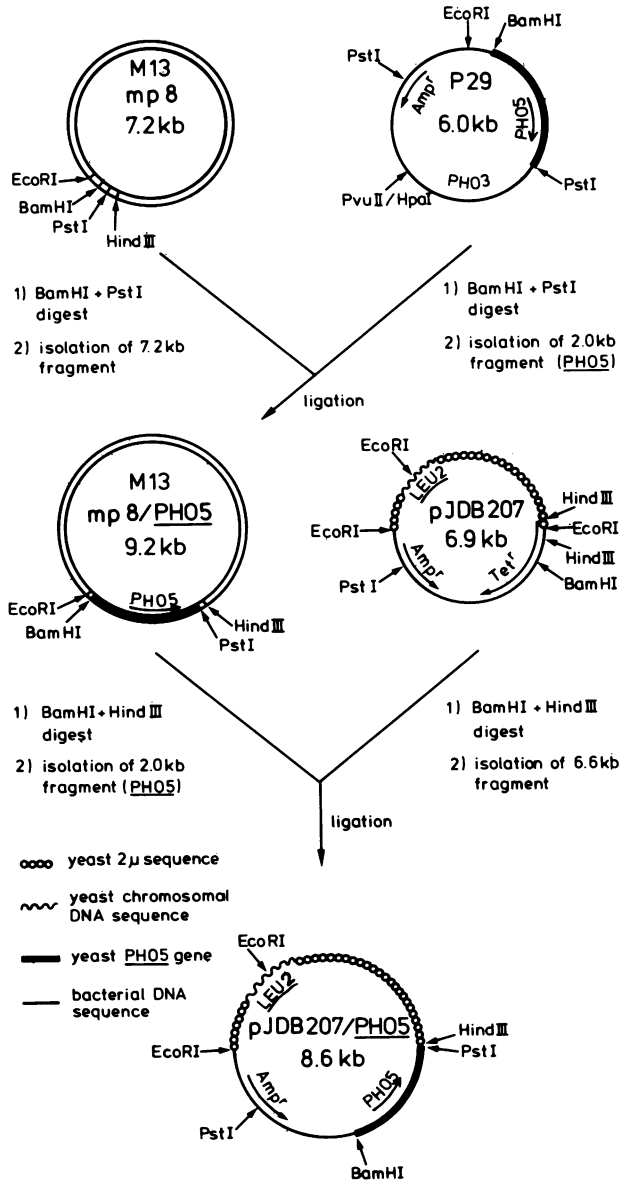

FIG. 2. Construction of expression plasmid pJDB207/*PHO5* (*Bam*HI-*Pst*I).

TABLE 1. Acid phosphatase activity of intact yeast cells transformed by plasmids carrying wild-type or deleted *PHO5* gene

Yeast strain	Plasmid	Growth medium	Acid phosphatase activity (U/OD600)[a]	Derepression in low-P_i medium (fold)	Increase due to presence of plasmid in derepressed cells (fold)
GRF18	—	High P_i	0.003		
		Low P_i	0.040	13	
GRF18	pJDB207/ *PHO5*	High P_i	0.15		
		Low P_i	0.62	4	15
GRF18	pJDB207/ *PHO5*/ΔK	High P_i	0.15		
		Low P_i	0.61	4	15
GRF18	pJDB207/ *PHO5*/ΔBS	High P_i	0.04		
		Low P_i	0.28	7	7

[a] Yeast strain GRF18 (with or without the plasmids indicated in the table) was grown to stationary phase in minimal medium with glucose as the carbon source and was inoculated at an OD_{600} of 0.05 into high-P_i or low-P_i medium. Growth was stopped when the OD_{600} reached 1.5. Acid phosphatase activity of whole cells in their growth medium was then measured.

We introduced the wild-type *PHO5* gene into the high-copy-number yeast plasmid pJDB207 (5) which carries the *LEU2* gene. A 2.0-kb *Bam*HI-*Pst*I fragment which contains the whole *PHO5* gene (promoter and structural gene) was isolated from plasmid p29 and cloned into M13mp8 (27). This step results in the addition of a *Hin*dIII site close to the *Pst*I site at the end of the gene (Fig. 2). The plasmid pJDB207 was cleaved with *Bam*HI and *Hin*dIII and was then ligated with the 2.0-kb *Bam*HI-*Hin*dIII restriction fragment of M13mp8/ *PHO5*. Plasmid pJDB207/*PHO5* was used in the construction of pJDB207/*PHO5* derivatives carrying the deletions ΔK and ΔBS. The 2.0-kb *Bam*HI-*Pst*I fragments of p29/*PHO5*/ ΔK and p29/*PHO5*/ΔBS were isolated, and each was ligated to a mixture of the two 6.1- and 6.8-kb *Bam*HI-*Pst*I fragments resulting from complete *Bam*HI and partial *Pst*I digests of pJDB207/*PHO5*. Transformation of *E. coli* strain HB101 and selection for Amp^R clones enabled us to obtain plasmids with the expected deletions (pJDB207/*PHO5*/ΔK and pJDB207/*PHO5*/ΔBS). Restriction map analysis of the two deleted plasmids confirmed the extent of the deletions and the presence or absence of the expected restriction sites (data not shown).

Acid phosphatase activity in yeast cells transformed with plasmids carrying *PHO5* and the *PHO5*/ΔK and *PHO5*/ΔBS deletions. The three plasmids bearing the *PHO5* gene, either wild type or deleted (ΔK, ΔBS), were used to transform GRF18, a Leu^- wild-type yeast strain for the phosphatase genes. Leu^+ clones were selected and tested for repressible acid phosphatase activity. None of the transformed strains exhibited any change in growth behavior. Acid phosphatase activity was measured in intact cells, since the substrate *p*-nitrophenylphosphate has free access to the cell wall and the periplasmic space. The activity was increased in the presence of any of the three plasmids, even after growth in high-P_i medium (Table 1). This suggests that the concentration of some regulatory component is not high enough to repress the system when the *PHO5* promoter is present on a high-copy-number plasmid. Under derepressed conditions in low-P_i medium, the presence of the plasmids pJDB207/*PHO5* and pJDB207/*PHO5*/ΔK induced a 15-fold increase of acid phosphatase activity. Under these conditions, acid phosphatase was estimated to represent ca. 5% of the total protein as based on the specific activity of the purified enzyme (41).

Results also show that the ΔBS deletion led to a smaller increase of activity as compared with the wild-type gene or the ΔK deletion (Table 1). To determine whether this smaller increase was caused by an incomplete secretion of the ΔBS deleted protein, the acid phosphatase activity was measured in toluene-permeabilized cells. Toluene treatment is known to impair plasma membrane permeability in *E. coli* (9) as well as in yeasts (43) and therefore leads to free access of the substrate to the cytoplasm. Transformants were shifted from minimal medium to low-P_i medium, and samples were taken at various times to permit the measurement of enzyme activity in intact whole cells, toluene-treated cells (Fig. 3A), and the supernatant medium (Fig. 3B). In the case of GRF18,

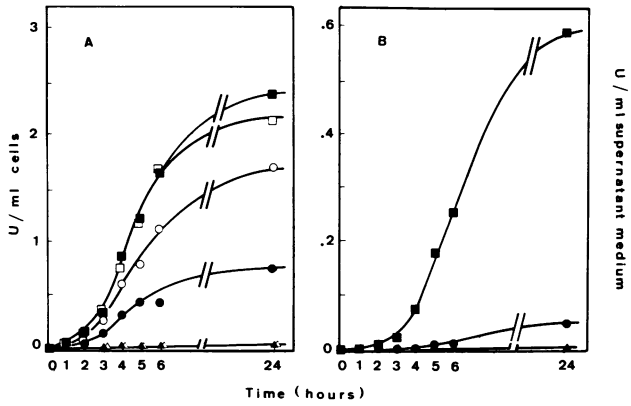

FIG. 3. Acid phosphatase activity in intact or toluene-treated cells (A) and in supernatant medium (B) after derepression of yeast cells transformed with *PHO5* and ΔBS plasmids. GRF18 nontransformed cells (▲, △) and cells transformed with *PHO5* (■, □) or ΔBS plasmids (●, ○) were grown up to stationary phase in minimal medium, harvested, and suspended at an OD_{600} of 1 in low-P_i medium supplemented with histidine and leucine (20 μg/ml; untransformed cells) or histidine alone (transformed cells). Samples were withdrawn at various time points during derepression and tested for acid phosphatase activity of intact cells (A; closed symbols), or toluene-treated cells (A; open symbols). Samples were centrifuged (3,000 × *g*, 2 min), and acid phosphatase activity of the resulting supernatant was measured (B). At the end of the experiment, the OD_{600} were 2.1, 2.3, and 2.2, respectively, for GRF18 control cells and cells transformed by *PHO5* and ΔBS plasmids.

the untransformed strain, and GRF18 transformed with the plasmid carrying the wild-type *PHO5* gene, the acid phosphatase activity of intact cells was identical to that of cells permeabilized with toluene (Fig. 3A). This was also the case for strains transformed by the plasmid pJDB207/*PHO5*/ΔK (data not shown). However, in the case of cells transformed by plasmid pJDB207/*PHO5*/ΔBS, the acid phosphatase activity of intact cells was half that measured in toluene-treated cells. Strains transformed by plasmid pJDB207/*PHO5*/ΔBS therefore produce an active phosphatase, only half of which reaches the cell envelopes even after 24 h of derepression. When GRF18 was transformed with the plasmid carrying the wild-type gene, ca. 25% of the acid phosphatase could be detected in the supernatant (Fig. 3A and B). This result was also obtained with the ΔK deleted acid phosphatase (data not shown). On the other hand, the ΔBS deletion led to a drastic decrease in the activity measured in the supernatant.

Processing of wild-type or deleted *PHO5* gene products in vivo and in vitro. The defect in secretion observed in the ΔBS deletion mutant was analyzed at the molecular level. Total RNA was prepared from the yeast strains transformed by plasmids carrying wild-type or deleted *PHO5* genes. In vitro translation products were analyzed after immunoprecipitation with an antibody reactive against acid phosphatase, followed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and fluorography. The relative molecular mass observed for the in vitro *PHO5* gene product (Fig. 4, lane 1) was ca. 58 kilodaltons (kDa) which corresponds to the 60-kDa polypeptide observed by others (7, 40). Both estimates are slightly higher than those deduced from the DNA sequence (1; Bajwa et al., submitted for publication). The ΔK and the ΔBS in vitro products (Fig. 4, lanes 4 and 7) are both 56 kDa, i.e., ca. 2 kDa smaller than the wild-type in vitro product; this corresponds with the reduction predicted by the DNA sequence. The intensity of the signals obtained by immunoprecipitation reveals that the amount of translatable RNA specific for acid phosphatase is similar in all three transformed strains. In comparison, the signal obtained for the in vitro product of RNA prepared from the derepressed untransformed strain was ca. 20 times less intense (data not shown) and was not detectable under the experimental conditions described in the legend to Fig. 4.

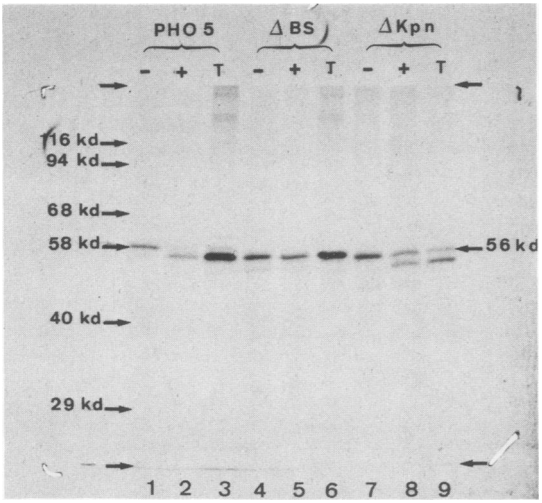

FIG. 4. In vivo and in vitro processing of *PHO5*, ΔK, and ΔBS gene products. Total RNA of transformed yeast cells grown and derepressed as described in the text was translated in a nuclease-treated reticulocyte lysate in the absence (−) or presence (+) of 40 μg of purified *E. coli* leader peptidase per ml. In vitro-translated proteins were submitted to immunoprecipitation, followed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and fluorography. Yeast cells grown and derepressed in low-P_i, low-S_i medium were treated by tunicamycin and labeled in vivo for 30 min in the presence of 500 μCi of [^{35}S]methionine per ml. Labeled cells were submitted to trichloroacetic acid precipitation and immunoprecipitation as described in the text (lanes T). Arrows at top and bottom indicate origin and front of the gel, respectively. The mobilities of molecular weight standards are indicated by arrows on the left side of the figure.

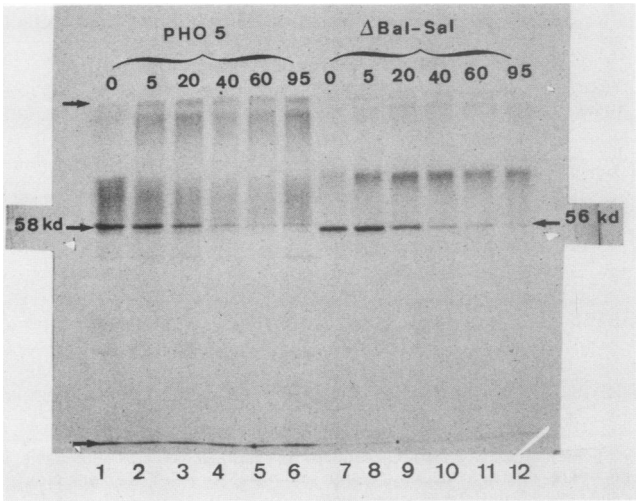

FIG. 5. Immunoprecipitation of acid phosphatase after pulse-chase labeling of intact yeast cells transformed with *PHO5* and ΔBS plasmids. Yeast cells grown and derepressed in low-P_i, low-S_i medium were submitted to a 5-min pulse labeling in the presence of 500 μCi of [^{35}S]methionine per ml, followed by a chase in the presence of 20 mM unlabeled methionine for the times indicated above each lane. Samples (200 μl) of total cells plus labeling medium were submitted to trichloracetic acid precipitation and immunoprecipitation. Lanes 1 to 6, *PHO5*-transformed cells; lanes 7 to 12, ΔBS-transformed cells. Arrows are as defined in the legend to Fig. 4.

In vivo proteolytic processing in all three transformed strains was studied in yeast strains labeled in the presence of tunicamycin, a compound which prevents *N*-asparagine-linked glycosylation of proteins (45) but not proteolytic processing of secretory proteins (13). The *PHO5* in vivo product was ca. 2 kDa smaller than the *PHO5* in vitro-translated product (Fig. 4, lane 3). Only trace amounts of the 58-kDa protein were detectable. Cells transformed with the *PHO5*/ΔK gene synthesized two polypeptides (Fig. 4, lane 9). One of these showed the same mobility as the corresponding in vitro product (56 kDa) and another was ca. 2 kDa smaller (54 kDa). In vivo and in vitro ΔBS products (Fig. 4, lanes 6 and 4) exhibited identical mobilities. These results may reflect complete, partial, and defective proteolytic processing, for the wild-type *PHO5* and ΔK and ΔBS polypeptides, respectively, which were formed in vivo in the presence of tunicamycin. A confirmation of these results was obtained by in vitro processing with purified *E. coli* leader peptidase (49). This enzyme removes the signal peptides from a wide variety of preproteins in bacteria and some preproteins in eucaryotes. The presence of leader peptidase during in vitro translation led to total, partial, or no proteolytic processing, for wild-type *PHO5*, ΔK, and ΔBS in vitro products, respectively (Fig. 4; lanes 2, 8, and 5).

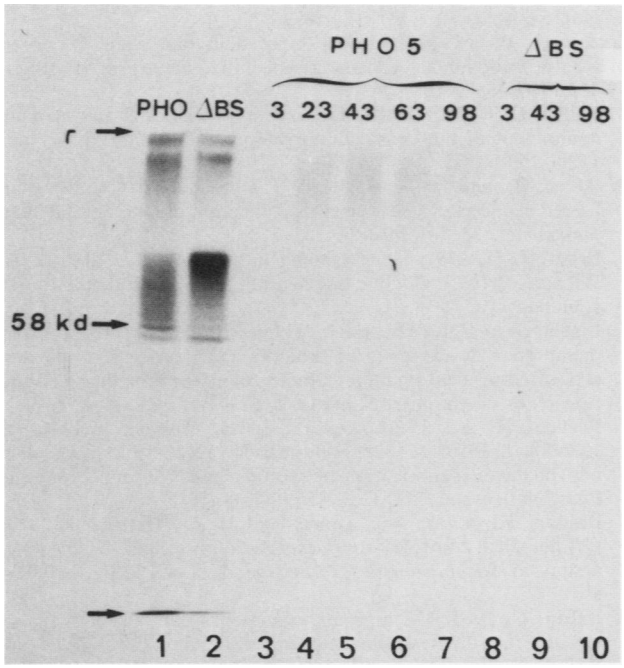

FIG. 6. Immunoprecipitation of acid phosphatase in the supernatant medium after a pulse-chase labeling experiment of yeast cells transformed with *PHO5* or ΔBS plasmid. Cells were labeled for 5 min and then chased with unlabeled methionine as described in the legend to Fig. 5. At the time points (min) indicated above the lanes, 200-μl samples were centrifuged (12,000 × *g*, 2 min), and the resulting supernatants were submitted to trichloroacetic acid precipitation and immunoprecipitation as described in the text. Lanes at left marked PHO and ΔBS correspond to 100 μl of total cells plus supernatant medium after 90 min of labeling. Arrows are as defined in the legend to Fig. 4.

In vivo glycosylation of overproduced wild-type *PHO5* and *PHO5*/ΔBS gene products. The effect of the ΔBS mutation on the glycosylation pattern of acid phosphatase was studied with in vivo labeling. Cells transformed by wild-type *PHO5* and *PHO5*/ΔBS plasmids were derepressed for acid phosphatase and submitted to a 5-min pulse in the presence of [^{35}S]methionine, followed by a long chase with unlabeled methionine (up to 90 min). Immunoprecipitation was performed in parallel on whole cells (Fig. 5) and on the supernatant (Fig. 6, lanes 3 to 10). The same culture was also labeled for a period of 90 min (Fig. 6, lanes 1 and 2). The 5-min labeling of *PHO5*-transformed cells led to an accumulation of unprocessed and unglycosylated acid phosphatase (58 kDa; Fig. 5, lane 1); processed and unglycosylated enzyme (56 kDa) was hardly detectable. At the beginning of the chase, several discrete bands of increasing molecular weight (up to 80 kDa) were clearly observed, this suggests various stages in core glycosylation. As the chase progressed, the quantity of both unprocessed and core-glycosylated immunoreactive polypeptides decreased, whereas high-molecular-weight species (100 to 140 kDa) showed a concomittant increase. These high-molecular-weight species most probably correspond to outer chain glycosylated polypeptides (15). Although very low amounts of the 56-kDa protein and of degraded proteins were detected by immunoprecipitation (Fig. 6, lane 1), the kinetics of the overall process suggest that the uncleaved unglycosylated polypeptide might be the precursor of the glycosylated polypeptides. Only the 100- to 140-kDa species were excreted into the medium in which they appeared after 23 min of chase (Fig. 6, lanes 3 to 7).

Glycosylation and secretion of ΔBS deleted acid phosphatase were significantly different from the corresponding events with the wild-type protein. A 5-min labeling of cells only led to the formation of unprocessed and unglycosylated polypeptide (Fig. 5, lane 7). A subsequent chase led to a progressive decrease in intensity of the unglycosylated polypeptide band, with an increase in core glycosylated polypeptides of mainly 80 kDa (Fig. 5, lanes 8 to 12). The amount of proteolytically degraded material was much less than in the *PHO5* case. For the *PHO5*/ΔBS-transformed cells, therefore, the 56-kDa polypeptide appeared as the precursor of the 80-kDa species. Fully glycosylated polypeptides were barely detectable in the pulse-chase experiment (Fig. 5, lanes 7 to 12) but were clearly visible after a 90-min labeling (Fig. 6, lane 2). They were characterized by a narrower range of molecular weights (120 kDa) than the corresponding wild-type glycoproteins. It might be argued that the high-molecular-weight glycosylated bands represent a wild-type acid phosphatase coded for by the chromosomal copy of the *PHO5* gene. However, this would be inconsistent with the relative abundance of these bands after a long labeling period, the apparently narrow range of their molecular weights, the absence of excretion of the corresponding proteins (Fig. 6, lanes 8 to 10), and the total absence of the 58-kDa in vitro product after in vitro translation of RNA from *PHO5*/ΔBS-transformed strains (Fig. 4, lane 4).

DISCUSSION

We have transformed the yeast organism *S. cerevisiae* with a high-copy-number plasmid carrying a repressible acid phosphatase gene (*PHO5*) either as a wild-type allele or as one of two mutant forms with small deletions in or near the signal peptide. The two mutations exhibit specific effects on processing, glycosylation, and secretion.

We have shown that the wild-type *PHO5* protein is produced in vitro as a precursor which is proteolytically processed to produce the mature form as has been shown for yeast invertase (10, 37). In vivo labeling of yeast strains carrying multiple copies of the wild-type *PHO5* gene led to the transient formation of a 58-kDa polypeptide which presumably represents the unprocessed and unglycosylated phosphatase precursor polypeptide. This 58-kDa preprotein is also detectable after inhibition of glycosylation by tunicamycin. This is unusual since in the case of secreted eucaryotic proteins, cotranslational proteolytic processing is believed to precede chain completion (21). The presence of acid phosphatase preprotein is probably a consequence of an overproduction of the protein, leading to the saturation of some component of the processing machinery such as the signal peptidase.

In vitro processing by bacterial signal peptidase under appropriate experimental conditions was found to be effective for wild-type *PHO5*-coded polypeptide, ineffective for *PHO5*/ΔBS-coded polypeptide, and partially effective for *PHO5*/ΔK-coded polypeptide. This bacterial enzyme has already been shown to be capable of cleaving some eucaryotic preproteins (49). Recently, it has also been shown that an antibody specific against bacterial leader peptidase specifically recognizes a yeast microsomal protein (39). The DNA sequence of the wild-type gene and the amino acid sequence of the mature protein (1, 46) suggest that the cleavage site lies between amino acids 17 and 18. This is consistent with the inability of leader peptidase to cleave the *PHO5*/ΔBS-coded polypeptide, which still possesses the first 14 of the 17 signal peptide amino acids, and is also

consistent with its ability to cleave the *PHO5*/ΔK-coded protein whose deletion begins at two amino acids after the putative cleavage site. Although these results do not specify exactly the features which characterize a signal peptidase cleavage site, they do suggest that one essential feature for specific cleavage resides in the five amino acids which separate the beginning of the two deletions. Of these, the alanine at the carboxy terminus of the signal peptide may play a critical role: it is in this position in numerous other signal peptides (30). The reduced efficiency of cleavage of the signal peptide displayed by the ΔK deleted protein suggests that the beginning of the mature protein sequence participates in the interaction between nascent acid phosphatase and signal peptidase as has been shown for an M13 coat protein mutant (30).

Within the first 5 min of in vivo synthesis, ΔBS deleted protein exhibits an increase of molecular weight from 56 kDa up to 80 kDa. As in the case of wild-type protein, this increase is completely prevented by tunicamycin, an antibiotic that blocks *N*-asparagine-linked glycosylation by inhibiting the formation of dolichyl-*N*-acetyl-glucosaminylpyrophosphate. Therefore, ΔBS deleted protein, which still contains two-thirds of the hydrophobic portion of the signal peptide, undergoes core glycosylation. This probably reflects the ability of the mutated protein to cross the ER membrane, since *N*-asparagine-linked core glycosylation is thought to occur on the cisternal surface of the ER (20, 44). This situation is very different from that observed with an influenza virus hemaglutinin lacking either 11 amino acids from the 16 amino acids of its signal peptide (42) or the whole signal peptide (16). In these cases, an intracellular accumulation of unglycosylated forms of these deleted proteins has been demonstrated.

In the ΔBS deletion, the absence of cleavage of the signal peptide severely impairs outer chain glycosylation and leads to an incomplete secretion of the protein: 50% of the mutated protein is found as an active enzyme inside the cell, even after 24 h of derepression; the other 50%, however, is secreted to the cell surface, although in a form which cannot cross the cell wall. This result is compatible with the observation that, in *E. coli*, half of a mutated prolipoprotein in which the signal peptide is not cleaved still reaches its destination in the bacterial outer membrane (23, 24). In this case, the absence of removal of the signal peptide leads to a decrease in the rate of secretion. Establishing whether this is also the case for the ΔBS deleted acid phosphatase should further our understanding of glycoprotein export as should the precise localization and characterization of the extracellular and intracellular forms of the ΔBS deleted proteins.

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