The *BOS1* Gene Encodes an Essential 27-kD Putative Membrane Protein That Is Required for Vesicular Transport from the ER to the Golgi Complex in Yeast

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Abstract. We recently described the identification of *BOS1* (Newman, A., J. Shim, and S. Ferro-Novick. 1990. *Mol. Cell. Biol.* 10:3405–3414.). *BOS1* is a gene that in multiple copy suppresses the growth and secretion defect of *bet1* and *sec22*, two mutants that disrupt transport from the ER to the Golgi complex in yeast. The ability of *BOS1* to specifically suppress mutants blocked at a particular stage of the secretory pathway suggested that this gene encodes a protein that functions in this process. The experiments presented in this study support this hypothesis. Specifically, the *BOS1* gene was found to be essential for cellular growth. Furthermore, cells depleted of the Bos1 protein fail to transport pro-α-factor and carboxypeptidase Y (CPY) to the Golgi apparatus. This defect in export leads to the accumulation of an extensive network of ER and small vesicles. DNA sequence analysis predicts that Bos1 is a 27-kD protein containing a putative membrane-spanning domain. This prediction is supported by differential centrifugation experiments. Thus, Bos1 appears to be a membrane protein that functions in conjunction with Bet1 and Sec22 to facilitate the transport of proteins at a step subsequent to translocation into the ER but before entry into the Golgi apparatus.

THE process of vesicle-mediated transport is complex and involves several events. Carrier vesicles, containing contents destined to traverse the secretory pathway, bud from a donor membrane. These vesicles are then targeted to their acceptor compartment where they bind to and fuse with this membrane to permit the mixing of contents (Palade, 1975). How a transport vesicle is formed and how it delivers its cargo to the correct acceptor organelle are pivotal questions regarding the mechanism of membrane traffic that remain unanswered. Although little is known about the molecular details underlying the budding, targeting, and fusion events, it is assumed that the regulation and specificity of these processes are ensured by proteins. Therefore, a prerequisite to addressing the mode of vesicular traffic is the identification and characterization of these components.

Classical genetic studies in the yeast *Saccharomyces cerevisiae* have defined 11 *SEC* and *BET* genes whose products are required for the transport of proteins from the lumen of the ER to the Golgi complex (Novick et al., 1980; Newman and Ferro-Novick, 1987). The *SEC* and *BET* gene products may participate in the budding of vesicular carriers from the ER or their subsequent binding and fusion with the Golgi apparatus. The recent development of assays that faithfully reproduce these events in vitro (Ruohola et al., 1988; Baker et al., 1988) should facilitate studies aimed at elucidating the function of the Sec and Bet proteins.

We have described the isolation of a new gene, *BOS1* (*b*et1 *o*ne *s*uppressor), that suppresses the growth and secretion defect of *bet1* and *sec22* (Newman et al., 1990). The ability of this gene to specifically suppress two different secretory mutants, that disrupt transport from the ER to the Golgi complex, suggests that its product functions in this process. In this report, we show that the *BOS1* gene encodes a 27-kD protein containing a putative membrane spanning domain. To test the function of Bos1, we have constructed a strain in which the synthesis of this protein is under the control of the regulatable *GAL1* promoter. Here we demonstrate that yeast cells depleted of Bos1 fail to transport pro-α-factor and carboxypeptidase Y to the Golgi complex. This defect in transport leads to an extensive accumulation of ER and patches of small vesicles. These findings support the hypothesis that the *BOS1* gene product is one of several proteins mediating transport at this stage of the secretory pathway.

Materials and Methods

Growth Conditions

Yeast cells were grown in YP[1] medium (1% Bacto-yeast extract and 2%

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1. *Abbreviations used in this paper*: CPY, carboxypeptidase Y; LB, Luria-Bertani medium; YP, 1% Bacto-yeast extract and 2% Bacto-peptone.

Table I. Yeast Strains

Strain	Genotype	Source
NY363	MAT*a/α, leu2-3, 112/+, ura3-52/ura3-52, his4-619*	P. Novick*
SFNY59	MAT*a, ura3-52*, pRB307 (2 μm, *URA3*)	This study
SFNY61	MAT*a, ura3-52*, pFNB61 (2 μm, *URA3, BOS1*)	This study
SFNY26-6A	MAT*α, his4-619*	This laboratory
NY447	MAT*α, ura3-52, GAL⁺, his4-619*	This study
JSY1	MAT*a, GAL⁺, his4-619, GAL1-BOS1*	This study
ANY113	MAT*α, ura3-52, his4-619, bet1-1*	This laboratory
NY432	MAT*α, ura3-52, sec18-1*	P. Novick*
SFNY85-1B	MAT*a, ura3-52, GAL⁺, bet1-1*	This study
SFNY86-7B	MAT*a, ura3-52, GAL⁺, sec22-3*	This study
SFNY87-4D	MAT*a his4-619, GAL⁺, bet1-1, GAL1-BOS1,*	This study
SFNY88-5C	MAT*α, his4-619, GAL⁺, sec22-3, GAL1-BOS1*	This study

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Bacto-peptone) with 2% glucose or in Wickerham's minimal medium (Wickerham, 1946) supplemented with the appropriate nutrients (20 μg/ml of histidine, 40 μg/ml of uracil). For sulfate-free minimal medium, all sulfate salts were replaced with chloride salts. JSY1 was grown in YP or minimal medium with 0.5% galactose and 2% raffinose. To change growth medium, cells were pelleted in a clinical tabletop centrifuge at room temperature and resuspended in fresh medium. Cell densities were measured in a 1-cm quartz cuvette at a wavelength of 599 nm in an LKB Biochrom Ultro Spec Plus spectrophotometer (LKB Instruments, Gaithersburg, MD).

Genetic Techniques and Constructions

All plasmids constructed were transformed into *Escherichia coli* (DH1) to amplify DNA. Ampicillin resistant transformants were selected in Luria-Bertani medium (LB) plates containing ampicillin (100 μg/ml). Plasmid DNA extracted from *E. coli* (Birnboim and Doly, 1979) was transformed into Ura⁻ yeast strains using lithium alkali cation treatment (Ito et al., 1983).

Several plasmid constructions were required to construct JSY1, a strain in which the *BOS1* gene was placed under the control of the regulatable *GAL1* promoter. The scheme used to construct this strain is summarized in Fig. 5. Plasmid pYG1 was constructed as follows: pNRB187 (Table II) was cut with Bam HI, the 3′ recessed end was filled in using the *E. coli* pol I Klenow fragment, and the vector was gel purified and treated with alkaline phosphatase. A 0.26-kb Taq I-Ava II fragment, containing the amino terminus of $BOS1^{-69\ to\ 195}$, was excised from pNF13 and blunt end-ligated into the Bam HI site of pNRB187. To construct pYG2, a 1.05-kb Eco RI fragment from pYG1, containing the $GAL1\text{-}BOS1^{-69\ to\ 164}$ fusion, was then inserted downstream from *URA3* into the Eco RI site of pNRB103. Plasmid pYG3 was constructed as follows: pYG2 was digested with Nhe I, the recessed 3′ end was filled in using Klenow and the vector fragment was gel purified and treated with alkaline phosphatase. A 1.7-kb Kpn I fragment, upstream of *BOS1*, was excised from pFN8 (Table II) and blunt end-ligated in the correct orientation into the Nhe I site of pYG2. Lastly, pYG3 was digested with BglII and partially digested with Eco RI to obtain the 4.0-kb Bgl II-Eco RI fragment shown in Fig. 5. This fragment was transformed into NY 477 and Ura⁺ transformants were selected.

Plasmid pTB1, which contains *TrpE* fused to an internal fragment of *BOS1*, was constructed as follows: pFN13 (Table II) and pATH10 were digested with Sau 3A and Bam HI, respectively, and then electrophoresed into an agarose gel. The 0.44-kb Sau3A fragment (nucleotide 329–769 of the *BOS1* gene) and the pATH10 vector were purified from an agarose gel and the purified fragments were religated and used to transform *E. coli* (DH1). The *TrpE-BOS1* fusion protein contains 147 of the 244 amino acids encoded by the *BOS1* gene product.

DNA Sequence Analysis and Homology Search

To determine the DNA sequence of the Kpn I-Nco I fragment which contains the *BOS1* gene, restriction fragments were cloned into the polylinker site of M13 phage derivatives (mp18 and mp19; Dale et al., 1985) and the nucleotide sequence was determined by the dideoxy chain termination method (Sanger et al., 1977) using the Sequenase DNA sequencing kit (United States Biochemicals Corp., Cleveland, OH). For each sequencing reaction the replicated DNA was labeled with 5 μCi of [α-^{35}S]dATP. All sequencing reactions were electrophoresed at 1,500 V on 8% polyacrylamide gels (40:1.3, acrylamide/bisacrylamide), containing 8 M urea, and the dried gels were exposed to Kodak XAR-5 film for 24 h. The *BOS1* sequence was compared with that of other proteins entered into the National Biomedical Research Foundation protein sequence library. The FASTA and

Table II. Plasmids

Plasmid	Derivation
pFN8	YCp50, *BOS1*; 4.2-kb SphI fragment from pAN105 (Newman et al., 1990) inserted in the Sph I site
pFN13	YCp50, *BOS1*; 2.2-kb Hinc II-Nco I fragment from pAN105 inserted in the Hind III (blunt) site of the vector
pFN14	YCp50, *BOS1*::*URA3* gene disruption; 1.1-kb Hind III fragment (*URA3*) inserted in the internal Eco RI (blunt) site of *BOS1* in pFN8
pTB1	pATH10, $TrpE\text{-}BOS1^{329\text{-}769}$ fusion; 0.44-kb Sau3A fragment from pFN13 inserted in the Bam HI site
pRB8	pBR322; 1.1-kb Hind III fragment (*URA3*) inserted in the Hind III site
pNRB103	YCp50, 1.1-kb Hind III fragment (*URA3*) inserted in the Hind III site
pNRB187	YCp50, with *GAL1* promoter; 0.82-kb Eco RI-Bam HI fragment inserted in the Eco RI-Bam HI site; expression under *GAL1* control by cloning into the Bam HI site
pYG1	YCp50, $GAL1\text{-}BOS1^{-69\ to\ 195}$ fusion, 0.26-kb Taq I-Ava II fragment (amino terminus of *BOS1*) from pFN13 inserted in the Bam HI (blunt) site of pNB187
pYG2	YCp50, $GAL1\text{-}BOS1^{-69\ to\ 164}$ fusion; 1.05-kb Eco RI fragment from pYG1 inserted in the Eco RI site of pNB103
pYG3	YCp50, $GAL1\text{-}BOS1^{-69\ to\ 164}$; 1.7-kb Kpn I fragment (upstream region of *BOS1*) from pFN8 inserted into the Nhe I (blunt) site of pYG2
pFNB61	2 μm, *BOS1*, 15-kb Sal I fragment from pAN105, in the Sal I site of vector, pRB307 (from the collection of D. Botstein, Stanford University)

FASTP programs were used in the ktup-2 mode to perform this analysis (Lipman and Pearson, 1985).

Preparation of the TrpE-Bosl Fusion Protein, Rabbit Immunization, and Affinity Purification of the Anti-Bosl Antibody

SFNB16 (DH1 transformed with pTB1) was grown as described previously by Goud et al. (1988). Cells from a 200 ml culture (OD_{599} ~0.5) were pelleted and washed with 50 ml of 20 mM Tris-HCl (pH 7.0). The pellet was resuspended in 5 ml of cracking buffer (10 mM sodium phosphate, pH 7.2, 1% SDS, 6 M urea, 1% β-mercaptoethanol) and incubated for 2 h at 37°C. DNA was precipitated from the sample by centrifuging the lysate for 30 min at 20,000 rpm in a Ti 50 rotor (Beckman Instruments, Inc., Palo Alto, CA). The supernatant was mixed with concentrated sample buffer (Laemmli, 1970) and electrophoresed on a preparative 8% SDS polyacrylamide gel. The gel was stained for protein and destained. The fusion protein was excised from the gel and the gel stripe, containing the fusion protein, was washed several times with distilled water over the course of 2 h. The sample was chopped into small cubes and the hybrid protein was electroeluted from the gel at 100 V for 12 h onto dialysis membrane (Hunkapillar et al., 1983). This procedure yielded ~500 μg of fusion protein in 0.5 ml of dialysis buffer which was stored at −20°C.

The immunization protocol used was essentially the same as described by Louvard et al. (1982). The serum was affinity purified on an Affigel column to which the TrpE-Bosl hybrid protein was cross-linked. The protocol used was described by Goud et al. (1988). This procedure yielded 1.5 mg of purified antibody in a volume of 2.5 ml.

In Vivo Labeling and Immunoprecipitation

Cells were grown to early exponential phase at 30°C in minimal medium containing 100 μM ammonium sulfate, 0.5% galactose, 2% raffinose, 40 μg/ml uracil, and 20 μg/ml L-histidine. To repress transcription from the *GAL1* promoter, JSY1 cells were pelleted and resuspended (OD_{599} = 0.025) in medium containing 2% glucose as the sole carbon source. After 12 h of growth at 30°C (approximately six doublings), the OD_{599} of JSY1 failed to increase. At this point, one OD_{599} of wild type and JSY1 cells were pelleted and resuspended in 0.5 ml of minimal medium, which was supplemented with 25 μM ammonium sulfate, 2% glucose, nutrients, and 200 μCi of [^{35}S]sulfate. The cells were incubated for 45 min at 30°C and lysates were prepared and processed for immunoprecipitation as described below.

The *bet* and *sec* mutants were grown to early exponential phase at 25°C in minimal medium supplemented with 100 μM ammonium sulfate and 2% glucose. One OD_{599} of cells were pelleted, resuspended in 0.5 ml of the same medium containing 25 μM ammonium sulfate and incubated for 15 min at 37°C. After this incubation, [^{35}S]sulfate (200 μCi) was added to the medium and the incubation was continued for 45 min. The radiolabeled cells were washed with 1 ml of cold 10 mM sodium azide, resuspended in spheroplast medium (1.4 M sorbitol, 50 mM potassium phosphate (pH 7.5), 10 mM sodium azide, and 36 mM β-mercaptoethanol) containing 1 U of zymolyase 100T and incubated for 1 h at 30°C. The spheroplasts were pelleted, lysed in 100 μl of 1% SDS, and heated to 100°C for 3 min. Lysates were diluted with 900 μl of dilution buffer (0.2 M sodium chloride, 12.5 mM potassium phosphate, pH 7.5, 2% Triton X-100) and centrifuged for 15 min in an eppendorf centrifuge at 4°C. The supernatant (800 μl) was removed and added to an Eppendorf tube containing 2 μl of the appropriate antibody. Protease inhibitor cocktail (Waters and Blobel, 1986) was added and the incubation was continued for 16 h at 4°C. The antigen–antibody complexes were precipitated with Protein A–Sepharose (60 μl of a 10% solution) during a 90-min incubation at 4°C and the beads were washed three times with urea wash buffer (2 M urea, 200 mM sodium chloride, 100 mM Tris pH 7.6 and 1% Triton X-100) and three times with 1% β-mercaptoethanol. The washed Protein A Sepharose beads were heated in Laemmli sample buffer at 100°C for 3 min and subjected to SDS-PAGE (10 or 12.5%). The samples were normalized to compare strains; the amount of sample loaded onto the gel was based on the [^{35}S]sulfate incorporated into the total lysate.

High molecular weight markers used for SDS-PAGE were 205-kD myosin, 116-kD β-galactosidase, 97.4-kD phosphorylase B, 66-kD BSA, 45-kD ovalbumin, and 29-kD carbonic anhydrase. Low molecular weight markers were 66-kD BSA, 45-kD ovalbumin, 36-kD glyceraldehyde-3-phosphate-dehydrogenase, 29-kD carbonic anhydrase, 24-kD trypsinogen, 20.1-kD trypsin inhibitor, and 14.2-kD α-lactoalbumin. Dried gels were exposed to preflashed Kodak XAR-5 film.

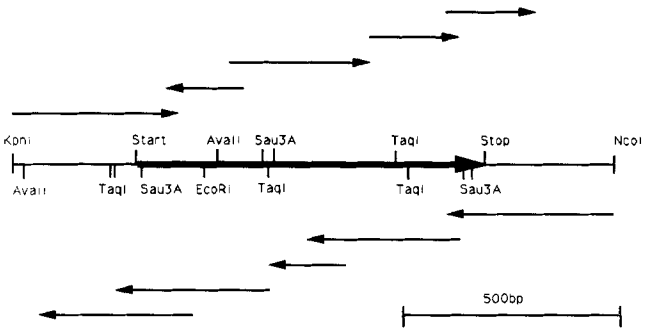

Figure 1. Restriction map of the Kpn I–Nco I fragment and sequencing strategy. The coding sequence of the *BOSI* gene is shown as a heavy black arrow. The putative intron, located immediately after the first codon, is stippled.

Results

Nucleotide Sequence of the BOS1 Gene

Our previous studies have shown that *BOSI*, *BETI* and *SEC22* are members of a group of interacting yeast genes (Newman et al., 1990). As a first step toward determining the function of Bosl, we cloned and sequenced the gene that encodes this protein. *BOSI* was identified by its ability to partially suppress the *betl* mutant at 37°C. Yeast genomic fragments containing this gene were described previously (Newman et al., 1990) and a 2.2-kb Hinc II–Nco I genomic insert, that suppressed *betl* to the same extent as the original isolate, was the smallest reported clone. This insert was subcloned to a 1.4-kb Kpn I–Nco I fragment and the nucleotide sequence of this region was determined by the strategy shown in Fig. 1. The 244-amino acid (27-kD) protein (called Bosl), contained within this fragment, suppressed the *betl* and *sec22* mutants (see Fig. 6). The *BOSI* gene contains a putative 88-bp intron with 5′(GTATGT) and 3′ consensus sequences for yeast RNA splicing (Langford et al., 1984; Padgett et al., 1986). Fig. 2 shows that this 5′ consensus splice site follows the first codon of *BOSI* and the yeast branch point box (TACTAAC) is 47 nucleotides downstream from the splice site. The predicted 3′ splice site is 25 nucleotides downstream from the branch point box.

DNA sequence analysis predicts that the amino terminus of the Bosl protein is hydrophilic with a hydrophobic stretch of 18 amino acids 4 amino acids from its carboxy terminus. This hydrophobic region is flanked by basic residues that may associate with negatively charged head groups of a phospholipid bilayer to anchor Bosl to a membrane. The DNA sequence also predicts one potential N-linked glycosylation site (at amino acid position 99) preceding the hydrophobic carboxy terminus. Since the primary sequence of Bosl does not contain a structure resembling a classical signal peptide, this protein may not enter the lumen of the ER where it would become accessible to the glycosylation machinery. Thus, this site may not be used. Further experiments will be needed before any final conclusions can be drawn.

BOS1 Is an Essential Gene

Since many of the genes required for protein transport perform an essential function, we have determined if *BOSI* is

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KpnI
-300      -290      -280      -270      -260      -250
GGTACCTAGTCCCTCTGGACCTTGGTGGGGTGGAAGGAGTCATTTTCGTGTAATAGTCCC
-240      -230      -220      -210      -200      -190
TGCTTCAAGGGTCAAAATTCCGTAGAGGTTTGGTTGATTGATTTACAAAGACAATACCTT
-180      -170      -160      -150      -140      -130
ATCCTTTGGATATGCCTTCTCTCTTTTTATAAATATCAAAAGTTGCGACGCGAACAACAA
-120      -110      -100       -90       -80       -70
GCCAGCCATTGAAATTTCAGTGTGCTGGCTATAATAGACAGGATCGACGCTGTCGATGGA
 -60       -50       -40       -30       -20       -10
GAAGGAAACACGGCAATAGTGCAGATATTATCGTGACTTAATCATATTGATTTGAGGGGG

   1        10        20        30        40        50
GA ATG GTATGTTTGATCGCGGAGGCATCTTTTCTCATTATTGCTAAAACAAGATGTAATA
  met ***************************************************

 60        70        80        90       100       110
CTAACAGGGTATTTTGCTACTACCATCAAATAG AAC GCT CTT TAC AAC CAT GCT GTG AAG
********************************* asn ala leu tyr asn his ala val lys

120       130       140       150       160 EcoRI  170
CAA AAA AAT CAA CTA CAA CAA GAG TTG GCC AGG TTT GAA AAG AAT TCT GTG ACC GCC CCT
gln lys asn gln leu gln gln glu leu ala arg phe glu lys asn ser val thr ala pro

180       190       200       210       220       230
ATT TCT TTA CAA GGG TCC ATC TCT GCA ACT CTG GTC TCA CTG GAG AAA ACA GTT AAG CAA
ile ser leu gln gly ser ile ser ala thr leu val ser leu glu lys thr val lys gln

240       250       260       270       280       290
TAT GCA GAA CAT TTA AAC AGA TAT AAA GAA GAT ACT AAT GCA GAG GAA ATT GAT CCT AAG
tyr ala glu his leu asn arg tyr lys glu asp thr asn ala glu glu ile asp pro lys

300       310       320       330       340       350
TTC GCT AAT CGA CTA GCA ACT TTA ACA CAG GAT CTG CAC GAC TTT ACT GCC AAG TTT AAG
phe ala asn arg leu ala thr leu thr gln asp leu his asp phe thr ala lys phe lys

360       370       380       390       400       410
GAT TTA AAA CAA TCC TAC AAC GAA AAT AAT TCC AGA ACT CAG TTG TTT GGC TCA GGA GCA
asp leu lys gln ser tyr asn glu asn asn ser arg thr gln leu phe gly ser gly ala

420       430       440       450       460       470
TCG CAT GTT ATG GAC TCC GAT AAC CCC TTT AGT ACA TCA GAG ACC ATC ATG AAT AAA AGG
ser his val met asp ser asp asn pro phe ser thr ser glu thr ile met asn lys arg

480       490       500       510       520       530
AAC GTT GGT GGT GCG AGT GCA AAT GGT AAA GAG GGC TCT AGC AAC GGT GGG GGA CTA CCG
asn val gly gly ala ser ala asn gly lys glu gly ser ser asn gly gly gly leu pro

540       550       560       570       580       590
TTG TAC CAA GGG CTA CAA AAG GAA CAG TCT GTT TTC GAA AGG GGT AAC GCT CAA TTA GAT
leu tyr gln gly leu gln lys glu gln ser val phe glu arg gly asn ala gln leu asp

600       610       620       630       640       650
TAC ATT CTA GAA ATG GGC CAA CAA TCA TTC GAA AAT ATA GTG GAA CAA AAC AAA ATT TTA
tyr ile leu glu met gly gln gln ser phe glu asn ile val glu gln asn lys ile leu

660       670       680       690       700       710
TCC AAG GTA CAA GAT AGA ATG TCA AAT GGC CTA AGA ACA TTG GGT GTT TCG GAA CAA ACT
ser lys val gln asp arg met ser asn gly leu arg thr leu gly val ser glu gln thr

720       730       740       750       760       770
ATC ACC TCT ATC AAT AAA CGG GTG TTC AAA GAT AAA CTA GTC TTT TGG ATC GCG TTA ATT
ile thr ser ile asn lys arg val phe lys asp lys leu val phe trp ile ala leu ile

780       790       800       810       820       830
CTC TTG ATC ATA GGT ATT TAT TAT GTG TTG AAA TGG TTA AGA TAG AAG TAA CCG TGA GTT
leu leu ile ile gly ile tyr tyr val leu lys trp leu arg stop  stop  stop

840       850       860       870       880       890
CTATATATAGATAAACATACCATATATACATGCATCATTATAAGGGTTGAAAAAACATCC
900       910       920       930       940       950
GAATCTCGCTACTTTCCTTCTATGCTCTTTCGTTTCTCAAACCCGCCAGTATAAGCAACT
960       970       980       990      1000      1010
TTTATTACCATTGGTGACTACACGTTGCTCCTTTTTTAAGTGTAATTTACTGTTATAAAT
1020      1030      1040      1050      1060      1070
TGAGTCCAAATCTGTCGTGTCTTGTCCTGGTTTGAGAACTTCGGGAATGTTCATTGCTTA
1080      1090      1100      1110  NcoI
GCGCATTTCATAGTTTTCTTTCCAATACCTAGTTTGGCCATGG
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Figure 2. DNA sequence of the Kpn I–Nco I fragment. The sequence of the 1.4-kb Kpn I–Nco I fragment, containing the *BOSI* gene, is shown. Promoter elements such as the upstream A:T stretch and TATA box are underlined. The intron is denoted by asterisks and the 5′ and 3′ splice sites as well as the branch point consensus sequences are in boldface. The amino acid sequence is shown below the nucleotide sequence. Lastly, a potential membrane-spanning domain of 18 hydrophobic amino acids, located at the COOH terminus of the predicted protein, is shown in boldface. These sequence data are available from EMBL/GenBank/DDBJ under accession number X57792.

needed for the vegetative growth of yeast cells. Plasmid pFN14 (described in Table II), containing a disrupted copy of the *BOSI* gene, was constructed in several steps. A 1.1 kb HindIII fragment, marked by the *URA3* gene, was excised from pRB8 (described in Table II) and inserted into pFN8

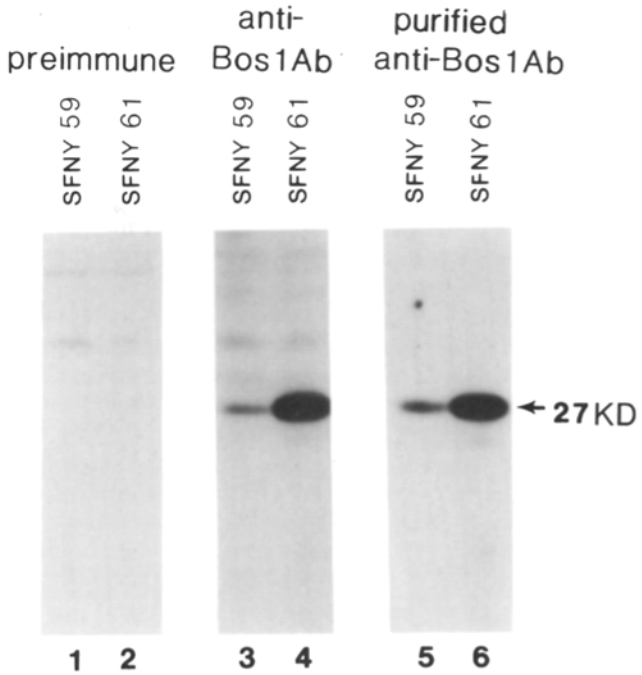

Figure 3. Antibody prepared to a TrpE–Bosl hybrid protein recognizes a 27-kD yeast protein. Cells transformed with a high-copy vector (SFNY 59) or with a high-copy vector containing the *BOSI* gene (SFNY 61) were broken with glass beads as described before (Ruohola et al., 1988). One OD_{599} unit of cells were subjected to electrophoresis on 12.5% SDS polyacrylamide slab gel and immunoblotted with preimmune serum (lanes *1* and *2*), immune serum (lanes *3* and *4*) and 5 μg/ml of affinity-purified antibody (lanes *5* and *6*).

(described in Fig. 5) at a unique Eco RI site (shown in Fig. 1) internal to the *BOSI* gene. A 5.4-kb Sph I fragment, containing the disrupted gene, was removed from pFN14 and introduced into NY 363 (Table I), a homozygous Ura$^-$ diploid yeast strain. This Sph I fragment recombined with one of two copies of the *BOSI* gene present in diploid cells, replacing one copy with the disrupted copy that contains *URA3*. Ura$^+$

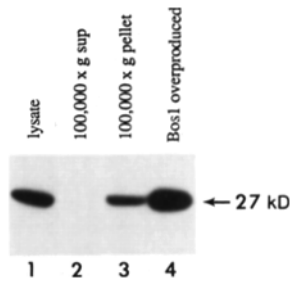

Figure 4. The Bosl protein sediments with yeast membranes. Wild-type yeast cells (200 OD_{599} units; SFNY26-6A) grown to early log phase in YPD medium were converted to spheroplasts and lysed as described before (Ruohola and Ferro-Novick, 1987). The lysate was centrifuged at 450 *g* for 5 min to remove nonlysed cells and the supernatant was centrifuged at 100,000 *g* for 1 h. The high-speed pellet was resuspended in the same volume as the supernatant and aliquots of each fraction were heated in sample buffer at 100°C. Samples were subjected to electrophoresis on a 12.5% SDS polyacrylamide slab gel and immunoblotted with 5 μg/ml of affinity-purified antibody. The lysate (lane *1*) and 100,000 *g* supernatant (lane *2*) were loaded at the same volume while the 100,000 *g* pellet (lane *3*) contained twice the volume. Lane *4* contains an aliquot of SFNY61 prepared as described in the legend to Fig. 3.

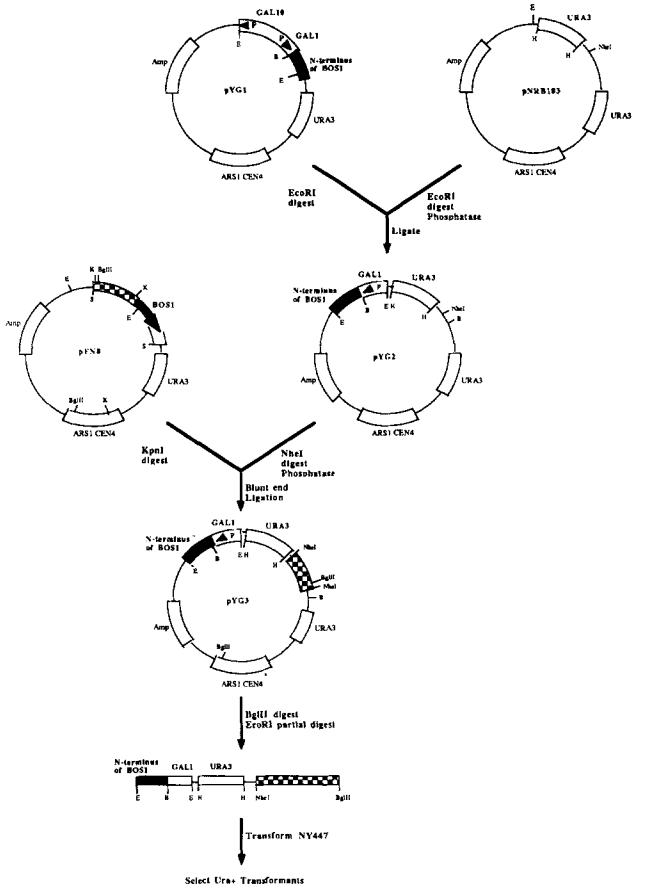

Figure 5. Construction of the *GAL1-BOS1* gene fusion. The strategy for constructing JSY1, a strain in which the *BOS1* gene is under the control of the regulatable *GAL1* promoter, is illustrated. First, to construct pYG1, the *BOS1* gene was placed behind the *GAL1* promoter (Johnston and Davis, 1984; Yocum et al., 1984) by inserting a TaqI-AvaII fragment containing a portion of *BOS1* into the BamHI site of pNRB187 (Table II). Then, an Eco RI fragment containing *BOS1* was excised from pYG1 and inserted into the Eco RI site of pNRB103 to yield pYG2. A Kpn I fragment, containing the region upstream from *BOS1* (*checkered*), was excised from pFN8 and inserted into the Nhe I site of pYG2 to yield pYG3. Finally, pYG3 was cut with Bgl II and partially digested with Eco RI. The resulting Eco RI–Bgl II fragment was transformed into NY447 and Ura$^+$ transformants were selected. *E*, Eco RI; *B*, Bam HI; *H*, Hind III; *S*, Sph I; *K*, Kpn I.

transformants were selected, sporulated, and twenty-two tetrads were dissected and germinated at 25°C. Two viable Ura$^-$ spores were recovered from each tetrad, implying that the Ura$^+$ spores were inviable. When examined under the light microscope, we noted that the inviable spores had initiated one round of cell division. These findings indicate that the *BOS1* gene is essential for the mitotic growth of yeast cells.

Identification of the Bos1 Protein and its Association with Microsomal Membranes

The nucleotide sequence of the *BOS1* gene predicts a protein of 27-kD. This prediction was tested by raising polyclonal antibody to a portion of Bos1 (amino acid 81–227) fused to the *E. coli* TrpE protein. Western blot analysis revealed that this antibody cross-reacts with a 27-kD species (Fig. 3, lanes *3* and *4*) which was not recognized by preimmune serum (Fig. 3, lanes *1* and *2*). Affinity-purified antibodies also cross-react with this species (Fig. 3, lanes *5* and *6*). To confirm that the 27-kD species is the Bos1 protein, we demonstrated that this immunoreactive protein was overproduced in a strain (SFNY61) harboring pFNB61, a high-copy plasmid containing the cloned *BOS1* gene (Fig. 3, lanes *2*, *4*, and *6*).

To determine if Bos1 is a soluble or membrane-associated protein, differential centrifugation studies were used. These experiments were performed with wild type yeast spheroplasts that were lysed in a buffer that provides osmotic support. The method of cell lysis used in these studies released 95% or more of a cytoplasmic marker protein (cytoplasmic invertase activity; Goldstein and Lampen, 1975). Nonlysed cells were removed from the homogenate by low-speed centrifugation (450 *g*) and the 450 *g* supernatant was centrifuged at 100,000 *g* for 1 h. Western blot analysis revealed that the Bos1 (Fig. 4, lane *4*), residing in the lysate (Fig. 4, lane *1*), sedimented during high-speed (100,000 *g*) centrifugation (Fig. 4, compare lanes *2* and *3*). However, on occasion, small amounts of this protein were found in the high speed supernatant (not shown). Thus, Bos1 appears to be associated with membranes. We have also performed extractions with sodium carbonate, a reagent known to solubilize proteins from the periphery of membranes (Howell and Palade, 1982). In an average of three experiments, we have found that 60% of the starting material remained in the pellet, 6% was solubilized, and the rest was not recovered, presumably because it was degraded. This observation supports the hypothesis that Bos1 is a membrane protein.

Construction of JSY1

We have demonstrated that the *BOS1* gene is essential for the vegetative growth of yeast cells. To determine the phenotypic consequences of depleting yeast cells of the Bos1 protein, we constructed a strain (JSY1) in which the only copy of *BOS1* was placed under the control of the regulatable *GAL1* promoter. In growth medium containing 2% glucose, the synthesis of Bos1 is repressed in this strain. To construct JSY1, we placed the *BOS1* gene behind the *GAL1* promoter and then inserted a piece of flanking DNA upstream from *BOS1* to facilitate replacement of the genomic copy with the inducible copy of this gene. This region of DNA was excised from the vector and then recombined into the genome. The manipulations involved in the construction of JSY1 are summarized in Fig. 5. Briefly, a Taq I–Ava II fragment, containing the amino-terminal portion of the *BOS1* gene, was inserted into pNRB187 (described in Table II) behind the *GAL1* promoter. The resulting *GAL1-BOS1* fusion was placed downstream from *URA3* by inserting an Eco RI fragment isolated from pYG1 into the Eco RI site of pNRB103. The region upstream of *BOS1* was inserted in front of the *URA3* gene by ligating the Kpn I fragment of pFN8 into the Nhe I site of pYG2. The resulting plasmid, pYG3, was digested with Bgl II and partially digested with Eco RI. The Eco RI–Bgl II fragment, containing the region upstream of *BOS1* and the *GAL1-BOS1* fusion, was transformed into NY447 and Ura$^+$ transformants were selected.

The growth properties of one transformant, JSY1, were examined on agar plates containing different carbon sources.

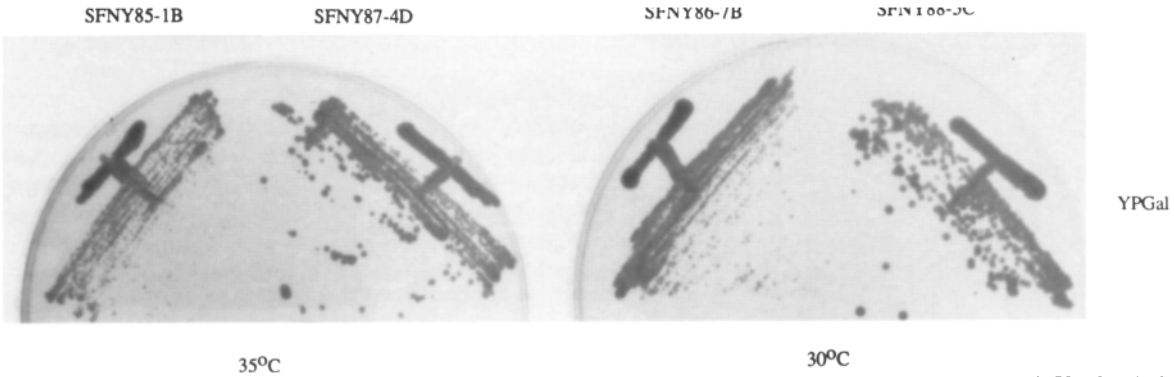

Figure 6. Gal-dependent suppression of *bet1* and *sec22*. At 35°C the *bet1* mutant, SFNY85-1B (MAT*a*, *ura3-52*, *GAL*$^+$, *bet1-1*), displays a partial defect in growth. This growth defect is suppressed when the Gal1–Bos1 fusion protein is expressed (SFNY87-4D). At 30°C the *sec22* mutant, SFNY86-7B (MAT*a*, *ura3-52*, *GAL*$^+$, *sec22-3*), displays a partial defect in growth. This growth defect is suppressed when the Gal1–Bos1 fusion protein is expressed (SFNY88-5C).

JSY1 failed to grow on YPD plates (YP + 2% glucose) but did grow as well as wild type (NY447) on YP plates supplemented with 2% raffinose and 0.5% galactose. A second transformant displayed the same properties. This result was anticipated since the *BOS1* gene is needed for the vegetative growth of yeast cells. To demonstrate that JSY1 expressed the Bos1 protein in a regulatable manner, we constructed haploid strains containing the *GAL1-BOS1* gene fusion in combination with either the *bet1-1* (SFNY87-4D) or *sec22-3* (SFNY88-5C) mutation. The ability of *BOS1* to suppress the growth defect associated with each mutation was then tested. We observed that the Gal1–Bos1 fusion protein suppressed *bet1-1* (SFNY87-4D) on YPGal (2% galactose) plates at 35°C, whereas *sec22-3* (SFNY88-5C) was suppressed at 30°C (Fig. 6). As expected, SFNY87-4D and SFNY88-5C failed to grow on YPD plates at 35°C and 30°C respectively (not shown). This suppression was specific to *bet1* and *sec22*, as the Gal1-Bos1 fusion protein failed to suppress *sec4* (not shown), a mutant blocked in post-Golgi secretion (Salminen and Novick, 1987).

Bos1-depleted Cells Fail to Transport Proteins to the Golgi Complex

The ability of the *BOS1* gene to specifically suppress the growth and secretion defect of mutants blocked in transit from the ER to the Golgi complex suggests that this gene encodes a protein that functions in this process. If the Bos1 protein is a necessary component of the yeast secretory apparatus, depletion of this gene product should block secretion. To test this prediction, we examined the transport of two different proteins in Bos1-depleted cells: a precursor of the yeast pheromone pro-α-factor and the vacuolar protease carboxypeptidase Y (CPY). To repress the synthesis of Bos1, ex-

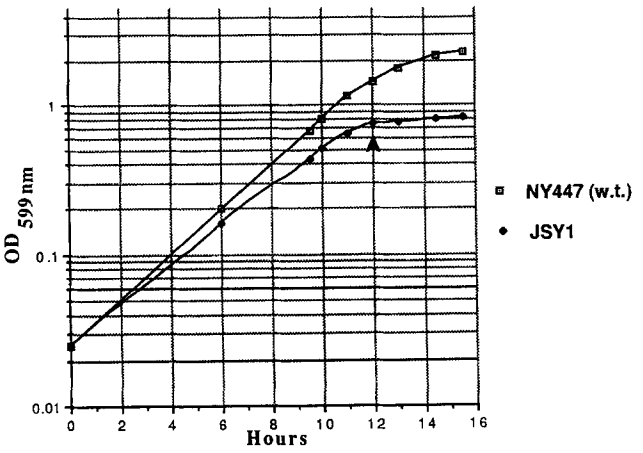

Figure 7. Growth of JSY1 and NY447 in minimal medium containing 2% glucose. JSY1 and wild type (NY447) cells were grown at 30°C to early exponential phase in minimal medium containing 100 μM ammonium sulfate, 0.5% galactose, 2% raffinose, 20 μg/ml histidine, and 40 μg/ml uracil. At the zero time point the cells were pelleted and resuspended in minimal medium containing 100 μM ammonium sulfate, 2% glucose, 20 μg/ml histidine, and 40 μg/ml uracil (OD_{599} = 0.025). After a 12-h incubation at 30°C, JSY1 failed to continue growing.

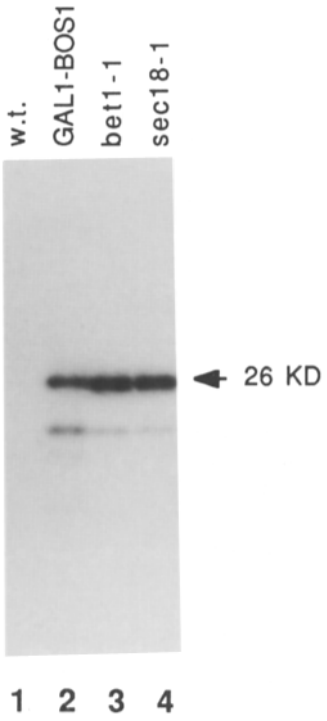

Figure 8. The 26-kD form of pro-α-factor accumulates in JSY1 when synthesis of the Bos1 protein is repressed. Synthesis of the *BOS1* gene product was repressed in JSY1 cells as described in Materials and Methods. One OD_{599} unit of cells, depleted of the Bos1 protein, was radiolabeled with 200 μCi of [^{35}S]sulfate for 45 min at 30°C. The *bet* and *sec* mutants were incubated for 15 min at 37°C and radiolabeled at this temperature for 45 min. Radiolabeled cells were converted to spheroplasts, lysed and pro-α-factor was immunoprecipitated. The solubilized immunoprecipitates were analyzed by SDS-PAGE (12.5%): (lane *1*) wild type (NY477); (lane *2*) *BOS1* under the control of the *GAL1* promoter (JSY1); (lane *3*) *bet1-1* (ANY 113); (lane *4*) *sec18-1* (NY432).

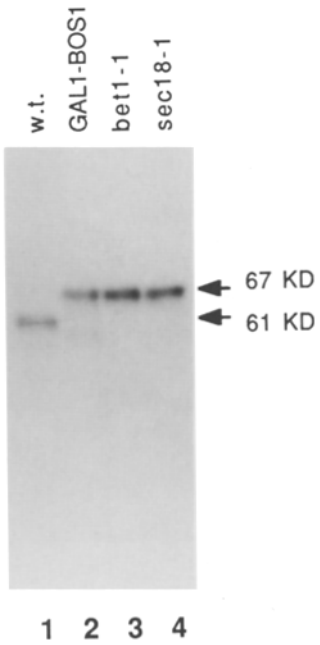

Figure 9. The pl form of CPY accumulates in JSY1 when synthesis of the Bosl protein is repressed. Cells were radiolabeled as described in Materials and Methods. The radiolabeled cells were converted to spheroplasts, lysed and the forms of CPY were immunoprecipitated and analyzed by SDS-PAGE (10%): (lane *1*) wild type (NY447); (lane *2*) *BOSI* under the control of the *GALI* promoter (JSY1); (lane *3*) *betI-1* (ANY 113); (lane *4*) *secI8-1* (NY432).

ponentially grown JSY1 cells were shifted into fresh medium containing glucose. After a 12-h incubation at 30°C, the OD_{599} of JSY1 failed to increase, whereas wild-type cells (NY447) continued to grow (Fig. 7). Lysates prepared from JSY1 cells, treated in this way, did not contain detectable amounts of the 27-kD Bosl protein when analyzed on Western blots (not shown).

The transit of pro-α-factor through the secretory pathway can be assessed by monitoring several processing events. The secreted pheromone α-factor, initially synthesized as a large 19-kD precursor molecule, contains a signal sequence, a hydrophilic proregion and four tandem repeats of the mature peptide (Kurjan and Herskowitz, 1982). As a consequence of signal sequence cleavage and the addition of three N-linked core oligosaccharide units, prepro-α-factor is converted to a 26-kD species in the lumen of the ER. These N-linked oligosaccharide chains are extended when pro-α-factor is transported to the Golgi complex. Pro-α-factor is then processed to the mature form in the *trans*-Golgi complex or in secretory vesicles enroute to the plasma membrane (Julius et al., 1984). To determine if the Bosl protein is required for pro-α-factor transport, JSY1 and wild-type cells (NY447) were labeled with [^{35}S]sulfate after a 12-h incubation in medium containing 2% glucose. Radiolabeled cells, converted to spheroplasts, were lysed in detergent and pro-α-factor was immunoprecipitated. Bosl-depleted cells accumulated the 26-kD species, as well as partially glycosylated forms of pro-α-factor (Fig. 8, lane *2*). These forms did not accumulate in wild-type cells (Fig. 8, lane *1*), but did accumulate in two previously identified mutants (*betI* and *secI8*) that block export from the ER to the Golgi complex (Fig. 8, lanes *3* and *4*). This finding indicates that yeast cells, lacking the Bosl protein, fail to transport pro-α-factor to the Golgi apparatus.

We also examined the processing of CPY in Bosl-depleted cells. ProCPY is synthesized as a zymogen with an 8-kD proregion and a cleavable signal sequence (Blachly-Dyson and Stevens, 1987; Hemmings et al., 1981). This protein acquires N-linked core oligosaccharides in the lumen of the ER, which are extended in the Golgi complex, before CPY is activated by the *PEP4* gene product in the vacuole (Hemmings et al., 1981; Stevens et al., 1982). Secretory mutants that disrupt protein export at or before the Golgi complex (Stevens et al., 1982; Newman and Ferro-Novick, 1987; Deshaies and Schekman, 1987; Toyn et al., 1988) fail to transport CPY to the vacuole. To determine if Bosl-depleted cells block CPY transport, lysates were prepared from cells radiolabeled with [^{35}S]sulfate as described above. JSY1, wild-type, and mutants cells, that block export from the ER to the Golgi complex, were examined. The *secI8* (Fig. 9, lane *4*) and *betI* mutants (Fig. 9, lane *3*) were found to accumulate the 67-kD pl form of CPY at 37°C (Stevens et al., 1982; Newman and Ferro-Novick, 1987). This form (Fig. 9, lane *2*) was also synthesized in Bosl-depleted cells, whereas the mature form (61-kD) was synthesized in wild type cells (Fig. 9, lane *1*). Therefore, in the absence of Bosl protein, CPY fails to be transported to the Golgi apparatus.

In the experiments described above, JSY1 cells were analyzed subsequent to a long incubation (12 h) in glucose containing growth medium. If the observed block in transport is a direct consequence of the loss of Bosl, the onset of this defect should correlate with the depletion of this protein. JSY1 and wild type cells were incubated for varying periods

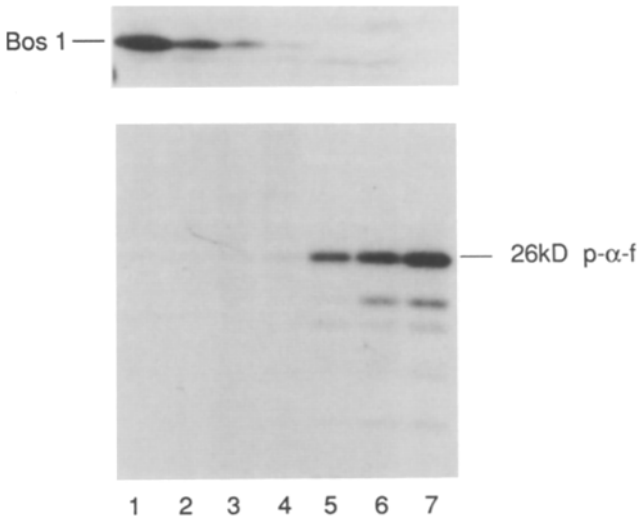

Figure 10. The depletion of Bosl protein from JSY1 cells is coincident with the block in secretion. Cells were grown overnight at 30°C in minimal medium containing 100 μM ammonium sulfate, 0.5% galactose, 2% raffinose, 40 μg/ml uracil, and 20 μg/ml L-histidine. Early log phase cells were resuspended in the same medium, containing 2% glucose, as described in Materials and Methods. Aliquots of cells (one OD_{599} unit) were removed at 0 (lane *1*), 2 (lane *2*), 4 (lane *3*), 6 (lane *4*), 8 (lane *5*), 10 (lane *6*), and 12 (lane *7*) h, analyzed for the presence of Bosl protein by Western blot analysis, and examined for a defect in pro-α-factor (p-α-f) transport, as described in the legend to Fig. 8 and Materials and Methods. A defect in pro-α-factor transport was detected at the 8-h time point, when cell viability was approximately the same as time zero. Cell viability was measured by plating an aliquot of cells onto YP plates, containing 0.5% galactose and 2% raffinose, and comparing the number of viable cells obtained to that of the zero time point.

of time (0, 2, 4, 6, 8, 10, 12 h) in glucose containing growth medium and the presence of Bosl protein was measured at each time point. At time zero, JSY1 cells contained approximately four times more Bosl protein than NY447 (not shown). Although the level of Bosl remained unaltered in wild type, this protein was depleted from JSY1 subsequent to 8 h in glucose containing medium (see Fig. 10 *A*, lane *5*). During this incubation, aliquots of cells were removed at various times, radiolabeled, and pro-α-factor transport was monitored as described above. A secretion defect was first observed at 8 h (Fig. 10 *B*, lane *5*), when the cells were viable and in exponential growth (Fig. 7). The same results were obtained when CPY transport was examined (not shown). Thus, the export defect observed in JSY1 cells correlates with the depletion of Bosl protein.

Bosl-depleted Cells Accumulate ER and Patches of Small Vesicles

The previously identified *sec* and *bet* mutants, that block transport from the ER to the Golgi complex, accumulate an extensive network of ER at their restrictive growth temperature. Vesicles, 40–60 nm in size, also accumulate in some of these mutants (Novick et al., 1980; Newman and Ferro-Novick, 1987). To determine the morphological consequences of depleting yeast cells of the Bosl protein, thin sections of cells lacking Bosl were examined by electron microscopy. In wild type, tubules of ER in contact with the nuclear envelope or at the periphery of cells were observed (Fig. 11 *A*). In contrast, JSY1 cells incubated for 10 h in glucose containing growth medium accumulated an extensive network of ER (Fig. 11 *B*). The lumen of the ER and the nuclear envelope were also dilated in these cells and patches of vesicles 50–60 nm in size were found in the cytoplasm (Fig. 11, *B* and *C*). These vesicles were comparable in size to the vesicles that accumulate in several other mutants (*bet1, bet2, sec17, sec18,* and *sec22*) blocked in transport from the ER to the Golgi complex (Novick et al., 1980; Newman and Ferro-Novick, 1987). Thus, thin section analysis confirms the findings discussed above and indicates that, in the absence of Bosl, protein transport is blocked before entry into the Golgi apparatus.

Discussion

In this report, we describe the characterization of *BOSI*, an essential gene identified as a stage-specific suppressor of ER-accumulating mutants (Newman et al., 1990). The ability of this gene to specifically suppress *bet1* and *sec22* suggests that its product acts in conjunction with the Bet1 and Sec22 proteins to perform an interrelated function. Thus, Bosl itself is likely to be required for ER to Golgi transport. To address this hypothesis, we have determined the phenotypic consequences of depleting yeast cells of the Bosl protein. This was achieved by constructing JSY1, a strain in which the sole copy of *BOSI* was placed under the control of the regulatable *GAL1* promoter. In growth medium containing glucose, JSY1 fails to synthesize Bosl. As a consequence, pro-α-factor transport is blocked and unprocessed precursors accumulate within the cell (Figs. 8 and 10). Cells depleted of the Bosl protein also fail to transport CPY to the vacuole (Fig. 9). The extent of precursor processing of proCPY and pro-α-factor is consistent with a block in ER to Golgi transport and

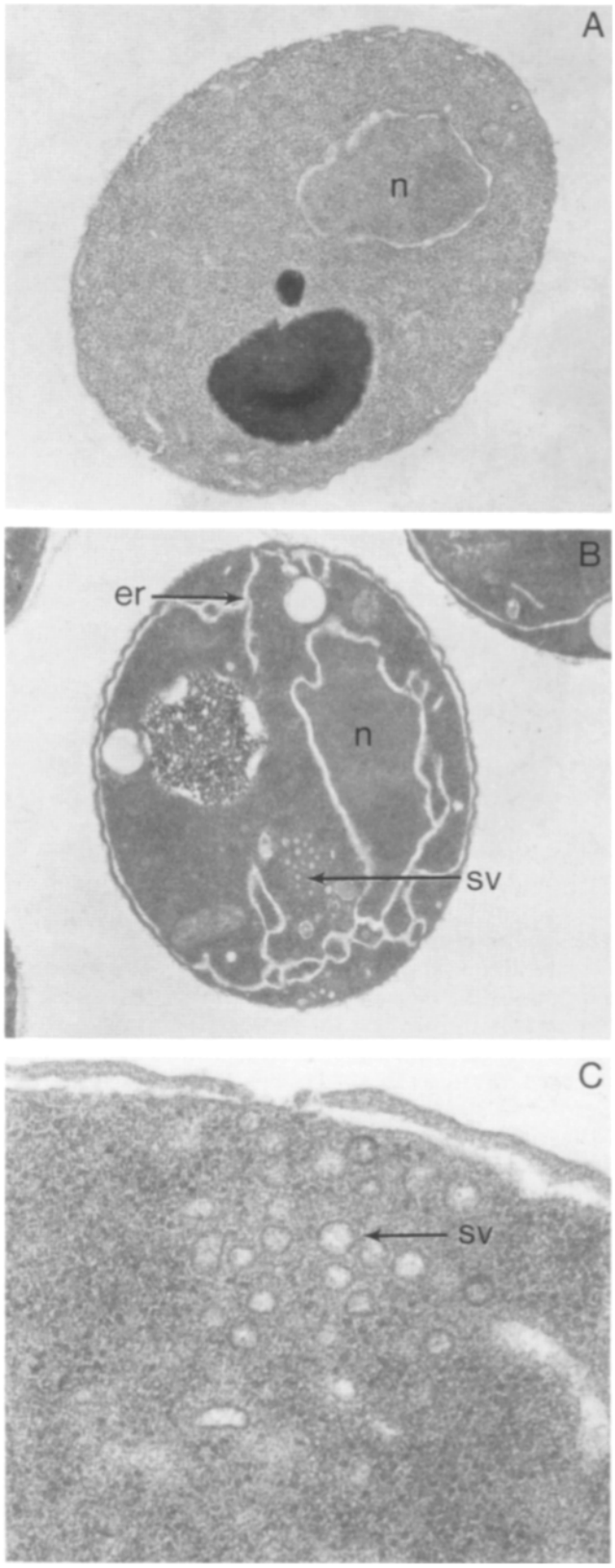

Figure 11. EM analysis of *BOSI*-depleted yeast cells. Samples were prepared for electron microscopic analysis as described by Newman and Ferro-Novick (1987). Thin-section analysis of wild-type (*A*) and *BOSI*-depleted cells, JSY1 (*B*). *C* is a high magnification of the small vesicles observed in Bosl-depleted cells. *er*, endoplasmic reticulum; *n*, nucleus; *sv*, small vesicles.

correlates with the loss of Bos1 protein (Fig. 10), supporting the notion that this gene product plays a direct role in secretion. Based on this observation, we anticipate that conditional lethal temperature-sensitive mutants in *bos1* should display a secretion defect within minutes after a shift to the restrictive temperature. This hypothesis will be tested, in the future, by constructing mutations in this gene.

Although no significant homologies were revealed when Bos1 was compared with other proteins in the National Biomedical Research Foundation protein sequence library, DNA sequence analysis has revealed that *BOS1* shares certain structural features with *BET1*. Both genes contain introns and encode small proteins that are hydrophilic at their amino-termini, with a stretch of hydrophobic amino acids at or near the carboxy end of the protein. The Bet1 protein is structurally similar to synaptobrevin, a constituent of synaptic vesicles (Newman, A., and S. Ferro-Novick, unpublished observations). Like synaptobrevin, Bet1 has significant alpha-helical potential in the middle region of the protein. In addition, a sequence of four amino acids "klkr," which is conserved in synaptobrevin homologues found in a variety of species, is also present in Bet1 at the appropriate distance from the hydrophobic carboxy terminus (Baumert et al., 1989; Sudhof et al., 1989). The *BOS1* gene does not, however, contain this consensus sequence.

Selection schemes previously used in yeast have led to the isolation of a large number of secretory mutants that disrupt vesicular transport at various stages of the pathway (Novick et al., 1980; Newman and Ferro-Novick, 1987). The distribution of mutant alleles, conferring a block in ER to Golgi transport, suggested that additional genes whose products mediate transport at this stage of the pathway remained to be identified. The *BOS1* gene was isolated (Newman et al., 1990) by its ability to suppress the growth defect of a known ER-accumulating mutant, *bet1* (Newman and Ferro-Novick, 1987). The isolation of *BOS1* and the characterization of its product has shown that suppression by overexpression is one means of identifying a gene whose product performs a function related to the gene bearing the original mutation. Several other examples have been reported whereby this tactic has led to the isolation of a gene that encodes a protein which functions in the same process (Salminen and Novick, 1987; Dietzel and Kurjan, 1987; Bender and Pringle, 1989; Nakano and Muramatsu, 1989). Thus, this approach provides a useful means of identifying new components that mediate secretion in yeast.

Does the 27-kD membrane protein encoded by the *BOS1* gene product mediate vesicle budding or subsequent stages of ER to Golgi transport? A morphological analysis of Bos1 depleted cells may provide a clue. Cells depleted of this protein accumulate a network of dilated ER (Fig. 11) as well as 50-nm vesicles. Vesicles of this size also accumulate in *sec17*, *sec18* and *sec22*, three mutants that disrupt transit from the ER to the Golgi complex (Novick et al., 1980). Recently, it was proposed that these 50 nm vesicles constitute an intermediate compartment that mediates transport at this stage of the pathway (Kaiser and Schekman, 1990). Thus, transport-incompetent vesicles, that fail to fuse with the Golgi apparatus, may bud from donor membranes in Bos1 depleted cells. Alternatively, Bos1 may control the attachment or fusion of intermediate transport vesicles with their acceptor compartment.

The yeast in vitro transport assay will enable us to address the role of the Bos1 protein in secretion. Transit from the ER to the Golgi complex is achieved in vitro when donor ER membranes are incubated with a yeast lysate in the presence of an ATP-regenerating system (Ruohola et al., 1988; Baker et al., 1988). This event is mediated by carrier vesicles, a short lived intermediate which is normally present in cells in low amounts. The ER to Golgi transport assay has enabled us to trap this intermediate in vitro, facilitating the isolation of this transient organelle (Groesch et al., 1990). Currently, we are purifying these vesicles to determine if Bos1 is a true constituent of this compartment. Now that we have developed an assay that permits the isolation of functional carrier vesicles, we can definitively determine whether the *BOS1* gene product controls vesicle budding or later stages of transport.

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