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Characterization of a Component of the Yeast Secretion Machinery: Identification of the *SEC18* Gene Product

KURT A. EAKLE,[1] MITCHELL BERNSTEIN,[2†] AND SCOTT D. EMR[1*]

Division of Biology, 147–75, California Institute of Technology, Pasadena, California 91125,[1] and Department of Biochemistry, University of California, Berkeley, California 94720[2]

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***SEC18* gene function is required for secretory protein transport between the endoplasmic reticulum (ER) and the Golgi complex. We cloned the *SEC18* gene by complementation of the *sec18-1* mutation. Gene disruption has shown that *SEC18* is essential for yeast cell growth. Sequence analysis of the gene revealed a 2,271-base-pair open reading frame which could code for a protein of 83.9 kilodaltons. The predicted protein sequence showed no significant similarity to other known protein sequences. In vitro transcription and translation of *SEC18* led to the synthesis of two proteins of approximately 84 and 82 kilodaltons. Antisera raised against a Sec18–β-galactosidase fusion protein also detected two proteins (collectively referred to as Sec18p) in extracts of ^{35}S-labeled yeast cells identical in size to those seen by in vitro translation. Mapping of the 5′ end of the *SEC18* mRNA revealed only one major start site for transcription, which indicates that the multiple forms of Sec18p do not arise from mRNAs with different 5′ ends. Results of pulse-chase experiments indicated that the two forms of Sec18p are not the result of posttranslational processing. We suggest that translation initiating at different in-frame AUG start codons is likely to account for the presence of two forms of Sec18p. Hydrophobicity analysis indicated that the proteins were hydrophilic in nature and lacked any region that would be predicted to serve as a signal sequence or transmembrane anchor. Although potential sites for N-linked glycosylation were present in the Sec18p sequence, the sizes of the in vivo *SEC18* gene products were unaffected by the drug tunicamycin, indicating that Sec18p does not enter the secretory pathway. These results suggest that Sec18p resides in the cell cytoplasm. While preliminary cell fractionation studies showed that Sec18p is not associated with the ER or Golgi complex, association with a 100,000 × *g* pellet fraction was observed. This suggests that Sec18p may bind transiently to small vesicles such as those presumed to participate in secretory protein transport between ER and the Golgi complex.**

The temperature-conditional yeast *sec* mutants define a set of functions required for secretory protein transport (20, 25). Nine *sec* genes, including *sec18*, have been shown to cause a block in the transport of secretory proteins between the yeast endoplasmic reticulum (ER) and the Golgi complex (7, 19). In *sec18-1* mutant yeast cells, secretory protein traffic is blocked at the level of the ER on a shift to the nonpermissive growth temperature (37°C). This is true for both secreted enzymes, such as invertase, and vacuolar proteases, like carboxypeptidase Y (CPY), which use the secretory pathway for intracellular localization (28). In *sec18* mutant cells, ER function per se is not impaired. Protein translocation across the ER membrane continues at the nonpermissive temperature, as do secretory protein processing activities such as signal sequence cleavage and core oligosaccharide addition. However, protein modifications characteristic of the Golgi complex, such as the addition of outer chain mannose residues, are not observed on the accumulated secretory proteins (7, 19). Electron microscopy has shown that *sec18* mutant cells accumulate exaggerated forms of the ER (19, 25). When *sec18* mutant cells are returned to the permissive temperature (23°C), accumulated secretory proteins undergo transport and processing through the remainder of the secretory pathway (20). Accumulated invertase is released at the cell surface as the normal, active, highly glycosylated enzyme. Together, the specificity of the *sec18-1* defect as well as its reversibility indicate that the *sec18* mutant accumulates an authentic intermediate in the protein secretion pathway. Because interorganelle traffic between secretory compartments likely occurs via vesicle carriers, the *SEC18* gene is presumed to encode a function that is involved in either the selective packaging of secretory proteins into carrier vesicles or the transport and fusion of such vesicles with the correct Golgi complex target membrane.

We report the cloning and sequencing of the *SEC18* gene. In addition, we characterized both the mRNA and two protein products (referred to collectively as Sec18p) encoded by this gene. Together, our observations suggest that Sec18p acts on the vesicle carriers that shuttle membrane and protein between ER and Golgi complex compartments.

MATERIALS AND METHODS

Strains. *Escherichia coli* Mc1061 [F$^-$ *araD139* Δ(*araABOIC-leu*)*7679* Δ*lacX74 galU galK rpsL hsdR*] (3) was used for cloning and fusion protein production, and JM101 was used for M13 phage growth for sequencing. *Saccharomyces cerevisiae* strains used were SEY2101 (*MAT***a** *ura3-52 leu2-3,112 suc2-Δ9 ade2-1*) (6), SEY5186 (*MATα sec18-1 ura3-52 leu2-3,112*) (5), and SEY6201 (*MAT***a** *ura3-52 leu2-3,112 his3-Δ200 trp1-Δ901 suc2-Δ9 ade2-101*) (this study). Strains were grown in standard medium preparations (16, 27).

Materials. Restriction enzymes, T4 DNA ligase, *Bal* 31 nuclease, mung bean nuclease, and 5-bromo-4-chloro-3-indolyl-β-D-galactoside (Xgal) were purchased from either New England BioLabs, Inc. (Beverly, Mass.) or Bethesda Research Laboratories (Gaithersburg, Md.) and were used

* Corresponding author.
† Present address: Department of Biology, Princeton University, Princeton, NJ 08544.

as recommended by the suppliers. Deoxy- and dideoxynucleotides were purchased from Pharmacia Fine Chemicals (Piscataway, N.J.). Universal sequencing primer was a gift from M. Simon. [^{32}P]dATP, [α-*thio*-^{35}S]dATP, $Na^{35}SO_4$, and Trans^{35}S label (a hydrolysate of *E. coli* grown in $^{35}SO_4$ containing ~70% [^{35}S]methionine and 20% [^{35}S] cysteine) were purchased from ICN Biochemicals (Irvine, Calif.). GeneScreen was purchased from Dupont, NEN Research Products (Boston, Mass.). Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) supplies and Western blot reagents (horseradish peroxidase [HRP] system) were from Bio-Rad Laboratories (Richmond, Calif.). The RNA cap analog [m^7G(5')ppp(5')G], ribonucleotides, and yeast translation extracts were gifts from E. Greyhack. SP6 RNA polymerase and RNasin were from Promega Biotech. Oxylyticase was purchased from Enzogenetics (Corvallis, Oreg.). Antiserum against β-galactosidase was a gift from T. Silhavy. Antiserum against the β subunit of mitochondrial F_1-ATPase was a gift from M. Douglas. Antiserum against CPY was from Klionsky et al. (10).

Recombinant DNA constructions. Preparation of DNA, restriction digestions, agarose gel electrophoresis, and cloning of DNA fragments were done as described previously (14).

DNA sequencing. DNA sequencing was done by standard dideoxy chain termination methods (24) by using *Bal* 31 deletion subclones of *SEC18* in M13mp19. DNA and protein sequences were compiled and analyzed with computer programs written by K. Eakle (unpublished data). Homology searches of the National Biomedical Research Foundation Protein Identification Resource data base were done on BIONET by using the XFASTP program (13).

Antisera production. *SEC18-lacZ* fusions were generated by cloning *Bal* 31 nuclease digestions of the 3.0-kilobase (kb) *Bam*HI-*Hin*dIII fragment into *Sma*I-cut pORF5. Clones were screened for overproduction of β-galactosidase activity on ampicillin-containing plates with Xgal. Plasmid DNA was prepared from candidate clones and checked for *SEC18* inserts by restriction mapping. Whole-cell extracts of *E. coli* with candidate plasmids were run on SDS-polyacrylamide gels (11) and evaluated by both Western blotting (30) with primary antisera against β-galactosidase (visualized by the HRP color reaction system) and staining with Coomassie blue to identify fusion protein bands and to estimate the extent of overproduction.

Fusion proteins were purified by growing *E. coli* with the fusion construction pORF18-40 to the stationary phase. Cells (250 OD_{600} units; 1 OD_{600} unit of cells equals the cells in 1 ml of a culture grown to an optical density at 600 nm of 1.0) were pelleted and suspended at 10 OD_{600} units per ml in 50 mM Tris (pH 8.0)–10 mM EDTA–10 mM dithiothreitol. Cells were lysed with a French press and spun at 6,000 × *g* for 20 min at 4°C in a rotor (JA-20), and the supernatant was spun at 170,000 × *g* for 30 min at 15°C in a rotor (Ti70.1). Membrane pellets were suspended in a small volume of buffer, and samples of the fractions were assayed for β-galactosidase activity at appropriate dilutions (16). Greater than 50% of the total β-galactosidase activity of the fusion proteins was associated with the membrane pellet, which yielded the equivalent of 2 to 3 mg of β-galactosidase activity. The largest fusion band was fragment purified from 5% SDS-polyacrylamide preparative gels that were stained with Coomassie blue. Commercially available β-galactosidase was also run on the preparative gels and was used to estimate the amount of fusion protein recovered. Fusion bands were homogenized, dialyzed against phosphate-buffered saline, and mixed with an equal volume of 1% agarose in phosphate-buffered saline. Approximately 50 μg of fusion protein was injected subcutaneously into young New Zealand White male rabbits weekly for 4 weeks, and then once every 2 weeks for 4 months. Antisera were initially evaluated for their ability to react in a Western blot procedure to the Sec18–β-galactosidase fusion protein that was present in a whole-cell SDS-polyacrylamide gel extract of *E. coli* Mc1061 transformed with the pORF18-40 fusion construct. Attempts at identifying the yeast *SEC18* gene product in a similar fashion from extracts of whole yeast cells by Western blotting were unsuccessful. Following identification of the Sec18 protein by in vitro translation, the antisera were found to specifically immunoprecipitate the [^{35}S]methionine-labeled in vitro translation product, as well as proteins with the same mobility on SDS-PAGE from extracts of yeast cells labeled with $^{35}SO_4$. A titration curve was generated for the *SEC18* antisera, as well as for the other antisera used in this study, by immunoprecipitating $^{35}SO_4$ labeled whole yeast cell extracts (1 OD_{600} unit per sample) with increasing amounts of antiserum. With this information, levels of antiserum were chosen which were in excess over the amount of the labeled proteins to be precipitated along with an amount of protein A-Sepharose CL4B sufficient to bind all the antiserum present, in order to ensure quantitative recovery. Precipitation with this same amount of antiserum against the Sec18p produced in yeast cells containing the *SEC18* gene on a multicopy plasmid with a 2μm origin of replication showed that this level of antiserum was sufficient to precipitate an eightfold excess of labeled *SEC18* gene product. Greater amounts of antiserum showed that the total level of Sec18p overproduction was about 20-fold with the *SEC18* gene on a multicopy plasmid.

Northern blot and 5′ and 3′ end mapping. Yeast RNA was prepared by a modification of a previously described procedure (4). Yeast cells were grown in YNB-glucose medium (27) to the mid-log phase. Yeast (50 OD_{600} units) were pelleted, washed with distilled H_2O, and suspended in 2 ml of 0.5 M NaCl–0.2 M Tris hydrochloride (pH 7.5)–10 mM EDTA–1% SDS. Cells were added to 15-ml polypropylene tubes with 2 g of acid-washed glass beads and 2 ml of phenol-$CHCl_3$ (1:1 [vol/vol]). Cells were chilled on ice, vortexed extensively, and spun at 12,000 × *g* at 4°C for 10 min in a rotor (JA-20). The supernatant was reextracted twice more with phenol-$CHCl_3$, and the RNA was precipitated with 2.5 volumes of cold ethanol. Pellets were washed with 70% ethanol, dried, and redissolved in 10 mM Tris hydrochloride (pH 7.5)–1 mM EDTA (TE). RNA recovery was measured by determining the optical density at 260 nm. Sodium acetate was added to 0.3 M, ethyl alcohol was added to 70%, and RNA was stored at −80°C. Poly(A)$^+$ RNA was isolated by binding to oligo(dT)-cellulose (Sigma Chemical Co., St. Louis, Mo.) in the presence of 0.5 M LiCl, washing extensively with the high salt buffer, and eluting with TE. RNA was run on formaldehyde–1% agarose gels and transferred to GeneScreen (14). Size standards were generated by ^{32}P end-labeling lambda DNA which was cut with *Eco*RI and *Hin*dIII. [^{32}P]RNA probes were made by transcribing the noncoding strand of our *SEC18* clone in pSP64 with SP6 RNA polymerase with [^{32}P]UTP and were hybridized to the blot as described previously (15).

Mapping at the 5′ end was done by using a ^{32}P-end-labeled oligonucleotide (5′-GGGAAATCATTTGGT-3′, complementary to bases 681 to 695 of the DNA sequence), which was hybridized to a single-stranded M13-*SEC18* subclone and extended with the Klenow fragment of *E. coli* DNA

polymerase I. The reaction was then digested with *Hin*dIII, and the probe was fragment purified on a 6 M urea–5% acrylamide gel. The probe (10^5 cpm) was hybridized to 25 μg of total yeast RNA in 80% formamide–40 mM PIPES [piperazine-*N*,*N*′-bis(2-ethanesulfonic acid); pH 6.8]–400 mM NaCl–1 mM EDTA in a total volume of 25 μl overlaid with 50 μl of mineral oil. The hybridization reaction mixture was heated at 85°C for 5 min and then incubated at 42°C overnight (12). The reaction mixture was diluted with 400 μl of 50 mM sodium acetate (pH 5.0)–10 mM NaCl–1 mM $ZnSO_4$ and digested with 50 U of mung bean nuclease for 1 h at 37°C (8). The reaction was phenol extracted twice and ethanol precipitated with glycogen as a carrier. To identify the size of the digestion products of the hybridization reaction, dideoxy sequencing reactions were carried out with the ^{32}P-end-labeled oligonucleotide as the primer and label in the reactions. The sequencing reactions and the digestion products of the hybridization reaction were run on a 6% sequencing gel.

Mapping at the 3′ end was performed as described above for the mapping of the 5′ end. The probe was a [^{32}P]UTP-labeled SP6 RNA polymerase transcript from the *Hin*dIII site of our *SEC18* clone to the *Pst*I site at position 2235. Hybridization was done as described above for 5′ end mapping, except that overnight incubation was done at 50°C. Samples were digested with mung bean nuclease as described above and run on a 5% sequencing gel, with ^{32}P-end-labeled lambda phage fragments and undigested probe used as size standards.

In vitro transcription and translation. Yeast in vitro translation extracts were prepared as described previously (31) and treated with micrococcal nuclease. RNA was generated by SP6 RNA polymerase runoff transcription of the minimum complementing subclone of *SEC18* cloned in pSP65, using ribonucleotide mixes with and without 0.5 mM $m^7G(5')ppp(5')G$ to give a capped mRNA (15). In vitro translation was performed as described previously (31) with 10 to 15 μCi of [^{35}S]methionine per reaction. Translation products were run on SDS–12% polyacrylamide gels (5% stacking gel). Gels were fixed, stained, treated with Auto-Flour (National Diagnostics, Somerville, N.J.), and dried before autoradiography on XAR film (Eastman Kodak Co., Rochester, N.Y.).

Immunoprecipitation. Yeast cells were grown to the mid-log phase in a defined medium (10) containing 100 μM SO_4. One OD_{600} unit of cells per immunoprecipitation sample were pelleted, washed with distilled H_2O, and suspended in 0.5 ml of sulfate-free medium–1 mg of bovine serum albumin per ml. Samples were incubated for 30 min at 30°C with shaking, and 100 to 200 μCi of $^{35}SO_4$ per OD_{600} unit of cells was added. Cells were labeled for 30 min at 30°C with shaking and unlabeled SO_4 was added to a final concentration of 50 mM. Labeling or chase was stopped by the addition of trichloroacetic acid to 5%, and samples were put on ice for 20 min. Samples were pelleted in a microfuge for 5 min, and the pellets were washed twice with cold acetone and dried. SDS boiling buffer (1% SDS, 50 mM Tris [pH 7.5], 1 mM EDTA, 100 μl per sample) and glass beads were added, and the samples were vortexed extensively and boiled for 3 min. One milliliter of Tween-IP buffer (50 mM Tris [pH 7.5], 150 mM NaCl, 0.1 mM EDTA, 0.5% [wt/vol] Tween 20) was added. Samples were vortexed and microfuged for 15 min in the cold. Supernatant (0.9 ml) was carefully removed, and 1 to 20 μl of the appropriate antiserum was added. Samples were rocked gently at 4°C for 3 h and 100 μl of 3.6% (wt/vol) protein A-Sepharose CL4B slurry was added. Samples were rocked for an additional 0.5 h at 4°C and pelleted. The pellets were washed (1 ml per wash) twice with Tween-IP buffer, twice with Tween-urea buffer (100 mM Tris [pH 7.5], 200 mM NaCl, 0.5% [wt/vol] Tween 20, 2 M urea), once with 1% β-mercaptoethanol, and once with 0.1% SDS. SDS-PAGE sample buffer (50 μl) was added, and the samples were boiled for 5 min before they were loaded onto an SDS-8% polyacrylamide gel (4% stacking gel).

Fractionation. Yeast cells to be labeled were pelleted, washed with distilled H_2O, and incubated with 100 mM Tris (pH 8.5)–25 mM dithiothreitol for 5 min at 30°C. Cells were pelleted, washed with distilled H_2O, and spheroplasted with 1 μg of oxylyticase per OD_{600} unit of cells in sulfate-free medium, which was adjusted to pH 7.5 and which contained 1.2 M sorbitol for osmotic support (22). Spheroplasts were pelleted gently, washed, suspended with sulfate-free medium (pH 5.5) containing 1.2 M sorbitol, and labeled with 50 to 100 μCi of Trans^{35}S label per OD_{600} unit of cells for 30 min at 30°C. Cells were pelleted at 500 × *g*, suspended gently in 100 μl of 1.2 M sorbitol–100 mM KPO_4 (pH 7.5)–10 mM EDTA, and lysed by rapid dilution with 1 ml of 0.25 M sucrose–10 mM Tris (pH 7.5)–10 mM EDTA–1 mM phenylmethylsulfonyl fluoride–1 mg of bovine serum albumin per ml. One OD_{600} unit of the lysed cells was precipitated with trichloroacetic acid for a whole-cell sample. Unlysed cells were pelleted at 2,000 × *g* for 2 min, and the supernatant was centrifuged at 13,000 × *g* for 15 min at 4°C. Supernatant (0.8 ml) from the microfuge spin was carefully removed and spun at 100,000 × *g* in a rotor (Ti70.1). Excess supernatant was removed carefully from the microfuge pellet, which was suspended in 100 μl of SDS boiling buffer, and boiled for 3 min. The supernatant from the 100,000 × *g* spin was precipitated with trichloroacetic acid on ice for 20 min, and the 100,000 × *g* pellet was suspended in 100 μl of SDS boiling buffer and boiled for 3 min. Samples were immunoprecipitated as described above.

RESULTS

Characterization and cloning of the *SEC18* locus. The *sec18-1* mutation in *S. cerevisiae* has been shown to cause a block in the transfer of proteins from the ER to the Golgi complex on a shift to the nonpermissive temperature. This block results in the accumulation of ER, as identified by thin-section electron microscopy (19, 20, 25). Proteins that transit the secretory pathway also accumulate with modifications that are consistent with their presence inside the ER, that is, with signal sequences removed and only core glycosylation units added to the protein backbone. This block is also reversible. The invertase that accumulated under these conditions has been shown to resume transport through the secretory pathway when it is returned to the permissive temperature (20). The speed with which ER transport is blocked by the *sec18-1* mutation is demonstrated in Fig. 1. Invertase was immunoprecipitated from *sec18-1* yeast cells that were grown under various conditions. In Fig. 1, lane 1 shows the size of unglycosylated invertase produced at the permissive temperature in the presence of tunicamycin. To determine the rate of onset of the block in secretory protein traffic in *sec18-1* mutant yeast cells, we shifted cells to 37°C for only 10 min before they were labeled with $^{35}SO_4$. Under these conditions, invertase accumulated as three distinct bands between 78,000 and 84,000 daltons (Fig. 1, lane 2). These bands corresponded to invertase, which varies in the total number of core oligosaccharide units added to the protein backbone (each core increases the apparent molec-

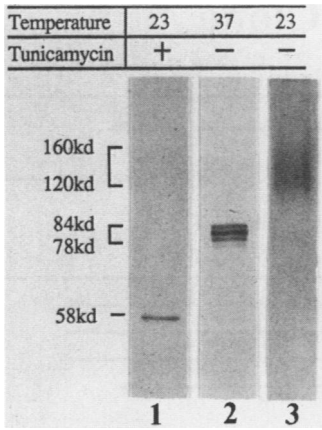

FIG. 1. *sec18-1* mutant cells were grown in 100 μM SO_4 medium with 2% glucose. Cells were pelleted, washed with distilled H_2O, and suspended in sulfate-free medium with 0.2% glucose for 30 min before they were labeled to induce invertase production. Cells were labeled with 0.5 mCi of $^{35}SO_4$ per ml for 20 min and chased for 20 min following the addition of $(NH_4)_2SO_4$ to 50 mM. kd, Kilodaltons.

ular weight of the protein by 2 to 2.5 kilodaltons). At the permissive temperature (Fig. 1, lane 3), *sec18-1* mutant cells process the labeled invertase to its highly glycosylated secreted form during passage through the Golgi complex. Thus, the *sec18-1* mutation results in a rapid block in secretory protein traffic at some stage after ER translocation and core oligosaccharide modification of secretory proteins.

Based on the results of morphology studies, the rapid and specific block in secretory protein movement, as well as the reversibility of the *sec18* mutant block, we reasoned that the *SEC18* gene is likely to code for a function that is directly involved in protein transfer between the ER and Golgi complex. To investigate this function, the *SEC18* gene was cloned by complementation of the *sec18-1* mutation with a genomic library of yeast DNA fragments in the multicopy vector YEp13 (18). Subcloning and further complementation analysis showed that the complementing activity resides on a 3.0-kb *Bam*HI-*Hin*dIII fragment. Because of a lack of restriction sites on one side of the clone, a library of *Bal* 31 deletion subclones was constructed which extended from either end of the *Bam*HI-*Hin*dIII fragment. Complementation analysis with these fragments cloned into the low-copy-number shuttle vector pSEYC58 (*CEN4 ARS1*) revealed that 500 base pairs (bp) of DNA at the *Bam*HI side of the fragment could be deleted without destroying the complementing activity (Fig. 2B). Deletion of as little as 200 bp from the *Hin*dIII side of the fragment was sufficient to destroy complementing activity.

To confirm that our clone carried the authentic *SEC18* gene and not a suppressor locus, we substituted the *LEU2* gene on a 2.1-kb *Hpa*I fragment for a 300-bp *Hpa*I fragment in the central region of the clone. A linear fragment with *SEC18* homology at both ends was transformed into the diploid strain SEY6201/SEY5186 (a/α *leu2-3, 112/leu2-3,112 SEC18/sec18-1*), and *Leu*$^+$ transformants were selected. Approximately 50% of the *Leu*$^+$ transformants simultaneously acquired the recessive Ts phenotype, indicating that the *SEC18* gene of one homolog was disrupted (23). Tetrad dissections of spores from the Ts diploid strains showed 2:2 segregation of *sec18*(Ts)/*Leu*$^-$: dead (*Leu*$^+$) spores per tetrad (n = 7). In addition, viable random spores from such diploids were all found to be *sec18*(Ts)/*Leu*$^-$ (200 haploid spores). These results indicate that our clone maps to the *SEC18* chromosomal locus and that *SEC18* is an essential gene. Examination of the dead spores showed that although many germinated and went through one cell division, none grew beyond the two-cell stage, suggesting that the intracellular pool of *SEC18* gene product is sufficient for spore germination but not large enough to sustain vegetative growth.

Further evidence that our clone contained the *SEC18* gene was obtained by transforming *sec18-1* mutant yeast cells with a yeast integrating plasmid (YIp5 vector) containing a noncomplementing fragment of the *SEC18* clone. This plasmid was restricted within the *SEC18* DNA to direct integration to the homologous chromosomal region (21). Following selection of *Ura*$^+$ transformants (all of which remained temperature sensitive), genomic DNA was prepared from integrants and restriction digested with an enzyme (*Eco*RI) which was expected to release the YIp5 vector together with adjacent *SEC18* sequences. The resulting DNA fragments were then ligated in dilute solution to recircularize the DNA, transformed into *E. coli*, and selected on the basis of ampicillin resistance. Restriction mapping of the resultant clones showed that they had, in fact, captured the entire *sec18* locus. These clones were shown to contain a functional temperature-sensitive allele of the *sec18* gene by transferring the gene to a yeast replicating vector (pSEYC58) and transformation into the *SEC18*::*LEU2*/*sec18*(Ts) diploid strain. These transformants remained temperature sensitive and on sporulation were able to give *Leu*$^+$ *Ura*$^+$ (Ts) haploids, indicating functional expression of a temperature-sensitive copy of *sec18* from the plasmid. To identify the approximate position of the temperature-sensitive lesion in the *sec18-1* mutant gene, restriction fragments were exchanged between the wild-type and *sec18*(Ts) clones. After transformation into *sec18-1* mutant yeast cells, the hybrid gene constructs were scored for whether they encoded a Ts or wild-type form of Sec18p. Based on results of these studies, the *sec18-1* mutation was found to unambiguously map to a 351-bp *Cla*I fragment (Fig. 2A). This placed the Ts mutation within the open reading frame of our *SEC18* clone.

DNA sequence of *SEC18*. The entire 3,042-bp *Bam*HI-*Hin*dIII *SEC18*-complementing DNA clone was sequenced by using standard dideoxy sequencing techniques, and the library of *Bal* 31 deletion subclones described above (Fig. 2C). The sequence (Fig. 3) revealed a 2,271-bp open reading frame which could potentially code for a protein product of 83,903 daltons. A search of the NBRF protein sequence data base showed no significant similarity between the predicted protein sequence and those of other known proteins (13). The position of the start of the smallest complementing subclone was only 35 bp upstream of the first ATG codon of the open reading frame, leaving little room for a *SEC18* promoter. Yeast gene promoters normally consist of a TATA sequence element that is usually 40 to 120 bases upstream from the site of transcription initiation and an additional enhancerlike element (upstream activating sequence) 5′ to the TATA box (29). Possible TATA sequences are found at positions 516 to 522 and 529 to 533, which are only 10 to 20 bp upstream of the open reading frame. No candidate sequences corresponding to an upstream activating sequence element in the region 5′ to the TATA boxes included in the smallest complementing clone were identified. Two additional ATG codons were present in the open reading frame 54 and 63 bp downstream of the first ATG codon. Combined, this information led us to question whether the transcriptional start point for the gene included the first ATG codon of the open reading frame.

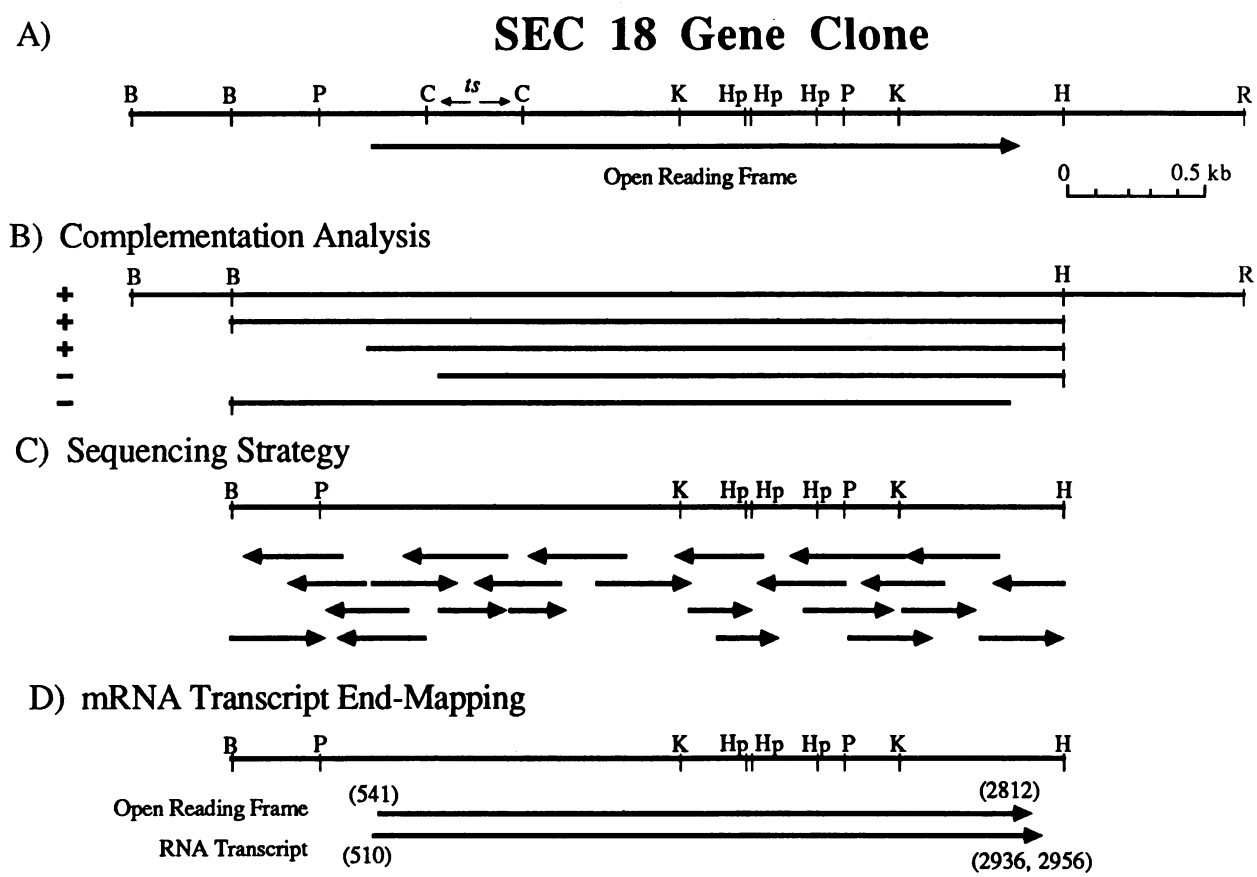

FIG. 2. (A) Restriction map of a *SEC18* clone showing the position of the open reading frame as determined by DNA sequencing. Restriction enzyme abbreviations: B, *Bam*HI; P, *Pst*I; K, *Kpn*I; Hp, *Hpa*I; H, *Hin*dIII; C, *Cla*I; R, *Eco*RI. The position of the mutation leading to the temperature-sensitive phenotype of *sec18-1* is indicated with double arrows. (B) Complementation analysis of *SEC18* subclones. Restriction fragments or *Bal* 31 deletions were cloned into pSEYC58 (*CEN4 ARS1 URA3*) and transformed into strain SEY5186 by LiCl yeast transformation. Transformants were selected for Ura$^+$ and tested for growth at 25 and 37°C. A plus sign indicates temperature-resistant transformants, and a minus sign indicates temperature-sensitive transformants. (C) Sequencing strategy used for DNA sequencing. Each arrow indicates independent subclones obtained by *Bal* 31 digestion of the 3.0-kb *Bam*HI-*Hin*dIII fragment from either end cloned into M13mp19. The entire *Bam*HI-*Hin*dIII *SEC18* fragment was cloned into M13mp8 and M13mp9 to obtain a sequence from the *Bam*HI site at the 5′ end of the clone and from the *Hin*dIII site at the 3′ end of the clone, respectively. (D) Comparison of the results of end mapping of the *SEC18* mRNA with the open reading frame of the DNA sequence. Numbers in parentheses represent the positions in the nucleotide sequence. The position of the 5′ end is mapped to ±2 nucleotides; the position of the 3′ ends are ±15 nucleotides.

Analysis of *SEC18* mRNA. Northern blots (Fig. 4A) with single-stranded probes from either strand of the *SEC18* clone confirmed that the only transcript from this region of DNA was a poly(A)$^+$, 2,500-nucleotide mRNA in the orientation of the open reading frame seen in the DNA sequence. End mapping experiments of the 5′ end of the *SEC18* transcript were done with a ^{32}P-end-labeled single-stranded probe and mung bean nuclease (Fig. 4B). Results of these experiments revealed that there is only one major initiation point for genomic transcription, beginning almost exactly at the position of the start of the smallest complementing subclone. This places our predicted TATA sequences within the transcribed region. Only the DNA which corresponded to the transcribed region appeared to be necessary for complementation of the *sec18*(Ts) defect. Because other single- and multicopy vectors were used to test complementation of this subclone, it seems unlikely that complementation was due to runon transcripts from other genes on our initial yeast shuttle vector. While aberrant expression of *SEC18* on a plasmid vector cannot be ruled out, the observation that the complementing subclone contained so little upstream sequence suggests that sequences which function in transcription initiation may be located within the transcript or may not be necessary for weak expression. The latter might be explained if only very low levels of *SEC18* transcription are required for complementation of the *sec18*(TS) defect.

End mapping of the 3′ end of the gene (data not shown) showed two major sites of transcription termination at approximately positions 2942 and 2956. Analysis of the sequence downstream from the termination of the open reading frame showed that this region conforms well to the consensus sequence ([T-rich region] . . . TAG . . . TAGT/TATGT . . . [AT-rich region]) for yeast termination and polyadenylation (32). The presence of three TAGT-like sequences suggests that these may function as two overlapping pairs of transcription terminators and, thus, lead to the two major sites of termination. Note also that both transcription termination sites were well beyond the TAA codon at position 2812, which would terminate translation of the open reading frames.

Identification of Sec18p in vitro and in vivo. The protein products of the *SEC18* gene were identified by in vitro translation with a yeast translation extract and an SP6 RNA polymerase-generated template RNA corresponding to the

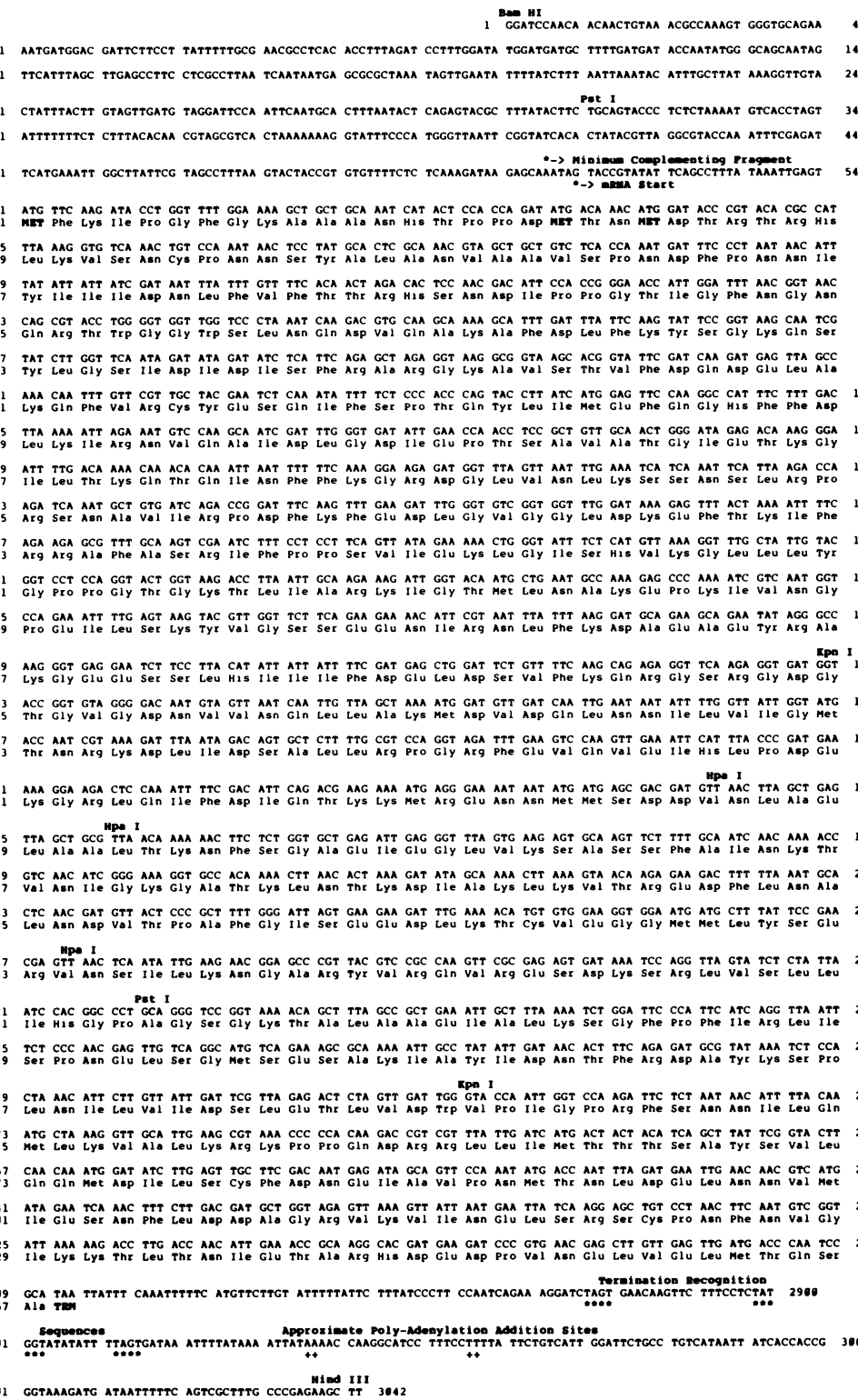

FIG. 3. Nucleotide sequence of *SEC18*. The nucleotide sequence with translation of the open reading frame. Positions of sites identified by restriction mapping prior to sequencing are indicated in boldface type, as are the positions of initiator methionine codons at the 5′ end of the open reading frame, the position of the start of the smallest complementing subclone, the position of the transcription start point for *SEC18* mRNA, the translation termination codon (TRM), and the transcription recognition sequences and approximate poly(A) addition sites at the 3′ end of the clone.

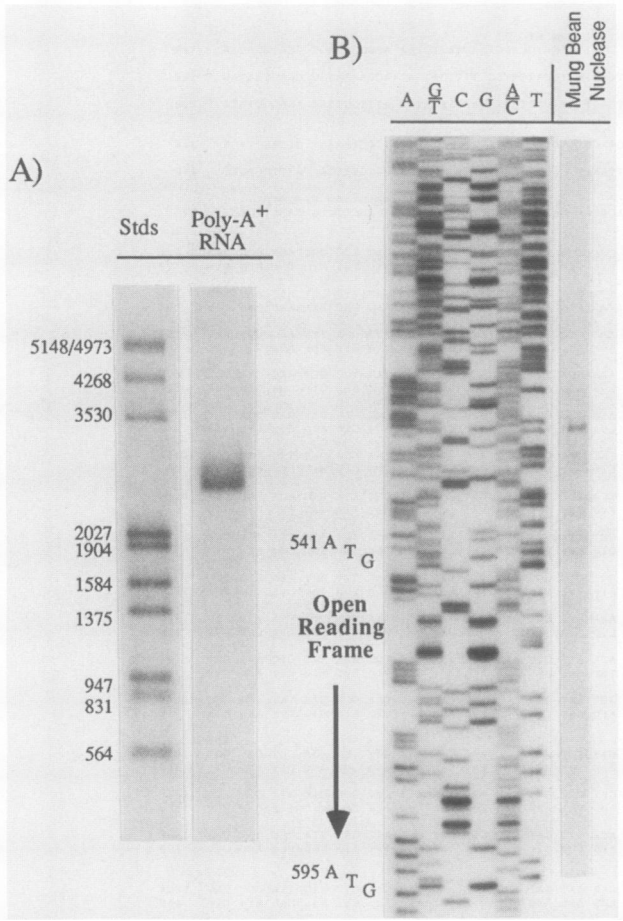

FIG. 4. (A) Northern blot of *SEC18* mRNA. Poly(A)$^+$ RNA (1 μg) from mid-log-phase SEY2101 cells was run on a 1% agarose-formaldehyde gel, transferred to GeneScreen, and probed with an anti-sense *SEC18* [^{32}P]RNA that was made with SP6 RNA polymerase. Standards (Stds) were *Eco*RI- and *Hin*dIII-cut lambda DNA that was end labeled by treating it with [γ-^{32}P]ATP and T4 polynucleotide kinase. Sizes (in nucleotides) are indicated to the left of the gel. (B) Mapping of the 5′ end of *SEC18* mRNA. An oligonucleotide complementary to bp 681 to 695 of the DNA sequence was end labeled with ^{32}P, hybridized to a single-stranded *SEC18*-M13mp19 template, and extended with the Klenow fragment to the *Bam*HI site. The single-stranded probe was fragment purified, hybridized to 25 μg of total RNA, and digested with mung bean nuclease. The DNA ladder was obtained by deoxy and dideoxy sequencing reactions by using the end-labeled oligo as primer. The positions of the first two ATG codons of the open reading frame of the DNA sequence are indicated to the left of the gel.

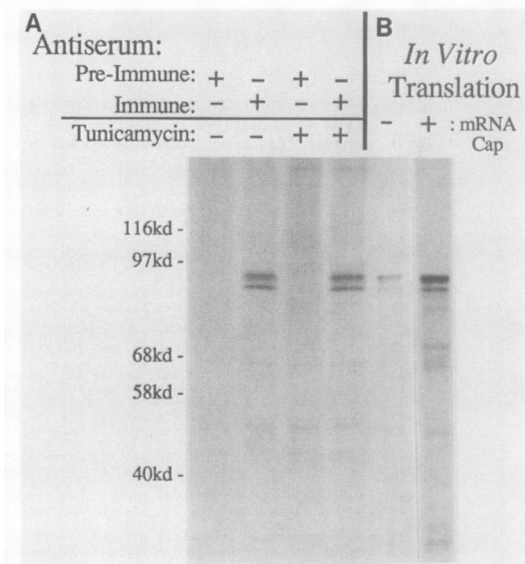

FIG. 5. Identification of Sec18p in vitro and in vivo. (A) Immunoprecipitations of Sec18p from ^{35}S-labeled whole-cell yeast extracts done with preimmune or immune serum in the presence or absence of tunicamycin, as indicated. (B) Products of in vitro translation of SP6 RNA polymerase transcripts of the *SEC18* open reading frame. RNAs were made with and without a mRNA cap analog [m^{7}G(5′)ppp(5′)G] in the transcription reaction, as indicated. Translation of the capped message proceeded with much higher efficiency. kd, Kilodaltons.

minimum complementing *SEC18* subclone. Two protein products (Sec18p) were detected, one of 84,000 daltons and one of 82,000 daltons (Fig. 5). The larger product corresponded well to the size predicted for translation of our entire open reading frame. The smaller translation product may have resulted from either premature termination of protein translation or initiation of translation at one of the two ATG codons located at positions 54 and 63 bp downstream from the start of the open reading frame. From the DNA sequence, these smaller open reading frames were predicted to encode proteins of 82,024 or 81,678 daltons. The yeast extract translation system that we used does not normally perform any posttranslational modifications of the protein products such as signal or prosequence cleavages or the addition of core oligosaccharides which could account for the multiple forms of the proteins that were produced. Transcription by SP6 RNA polymerase also has high fidelity in terms of the site of transcription initiation, so variation in the 5′ end of the RNA template does not seem likely (15).

In order to test whether the protein(s) produced from the *SEC18* open reading frame was also produced in vivo, we raised antisera to the *SEC18* protein. A gene fusion of the *SEC18* open reading frame to the 5′ end of the *E. coli lacZ* gene was generated by ligating random *Bal* 31 digestions of the 3.0-kb *Bam*HI-*Hin*dIII fragment into the vector pORF5 (26) (Fig. 6A). Clones which overproduced β-galactosidase activity were initially picked by screening on Xgal plates. Plasmids from these clones were screened for inserts corresponding to the *SEC18* gene by restriction site analysis. In addition, SDS-PAGE of whole-cell *E. coli* extracts expressing high levels of β-galactosidase activity were stained with Coomassie blue to visualize the extent of overproduction and to identify the size of the hybrid proteins (Fig. 6B). Western blotting of these gels with β-galactosidase-specific antibodies confirmed the identity of hybrid protein bands. Several gene fusions were created which overproduced fusion proteins in *E. coli* that were up to 40,000 daltons larger than β-galactosidase itself. The fusion joints between five of these clones and the *lacZ* sequences were determined by DNA sequencing, which confirmed that the large open reading frame of *SEC18* was being used. The largest fusion protein was purified by using preparative SDS-PAGE and was injected into rabbits to raise antiserum that was reactive to Sec18p. Antisera were tested initially for reaction to the Sec18–β-galactosidase fusion protein on Western blots and were later confirmed to react with the in vitro translation products of *SEC18*.

Immunoprecipitation from extracts of ^{35}S-labeled whole yeast cells with the Sec18p antiserum was able to detect in

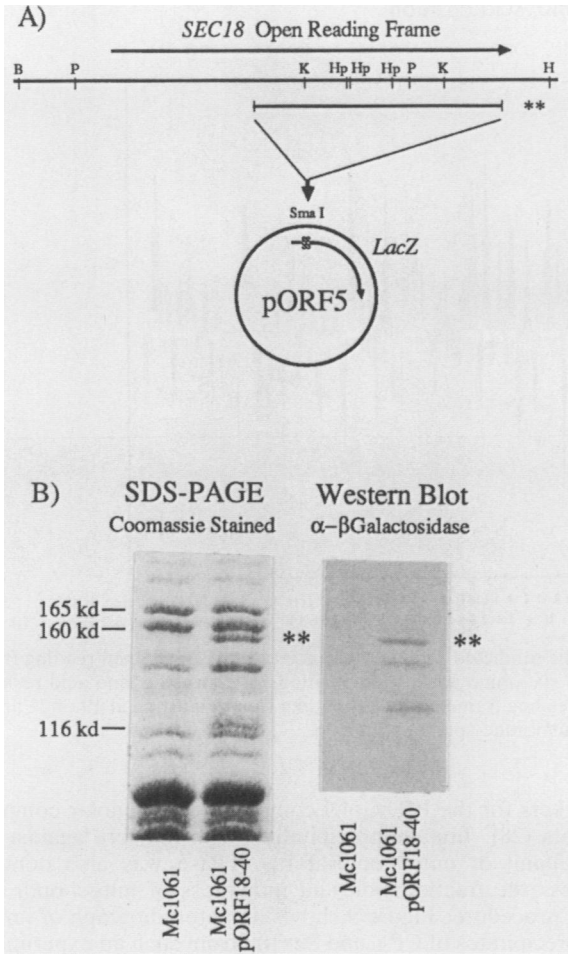

FIG. 6. Production of *SEC18-lacZ* fusion proteins. (A) *Bal* 31 digestions of the 3.0-kb *Bam*HI-*Hin*dIII fragment were cloned into the *Sma*I site of pORF5, a *lacZ* fusion vector in which a *Eco*RI-*Sma*I-*Bam*HI polylinker was inserted at the amino acid at position 7 to interrupt the reading frame of the *lacZ* gene. The fragment marked with double asterisks is clone pORF18-40, which expressed the largest fusion protein observed. Restriction enzyme abbreviations: B, *Bam*HI; P, *Pst*I; K, *Kpn*I; Hp, *Hpa*I; H, *Hin*dIII. (B) Whole-cell extracts of *E. coli* Mc1061, with and without the pORF18-40 fusion plasmid, were run on SDS-polyacrylamide gels. A gel stained with Coomassie blue G-250 is shown on the left, and on the right is a Western blot of a gel identical to that shown on the left whose proteins were transferred to nitrocellulose by Western blot and probed with anti-β-galactosidase antisera. The largest fusion protein is marked with double asterisks. kd, Kilodaltons.

vivo production of the putative *SEC18* gene products. The in vivo forms of Sec18p were identical in size to those seen by in vitro translation (Fig. 5). Tunicamycin, which inhibits N-linked glycosylation, had no effect on the size of the in vivo proteins that were observed, even though potential sites for glycosylation were present in the predicted amino acid sequence at amino acid positions 13, 36, 455, 474, and 689. This suggests that Sec18p is not translocated across the ER membrane into the secretory pathway, and thus, it never comes into contact with the oligosaccharide transferase enzyme. This was further supported by the hydrophobicity profile for the predicted Sec18p sequence (Fig. 7), which showed no evidence for a characteristic hydrophobic signal sequence at the amino terminus of either the full-length protein or a protein whose translation began at one of the two downstream ATG codons at the 5′ end of the open reading frame. In addition, the hydrophobicity profile did not show any internal regions which would be predicted to span a membrane or function as internal signal sequences. These data are most consistent with the fact that Sec18p remained in the cell cytoplasm. However, these results do not exclude the association of Sec18p with the cytoplasmic face of an intracellular organelle membrane.

The two forms of Sec18p seen in vivo also could have resulted from a posttranslational processing event other than glycosylation. To address this question, yeast cells were pulse-labeled for 20 min with $^{35}SO_4$ and chased for various times following the addition of excess cold SO_4. Whole-cell extracts were immunoprecipitated with antisera directed against both Sec18p and CPY, a vacuolar protease which undergoes processing in the ER, Golgi complex, and vacuole to give forms which can be distinguished on SDS-polyacrylamide gels (28). The results (Fig. 8) indicated that while CPY is processed normally with a half-time of approximately 5 min, the two forms of Sec18p are maintained in a constant ratio. This was true for chase periods of up to 2 h (data not shown). Thus, the two forms of Sec18p do not appear to result from posttranslational processing of the larger form into the smaller form or vice versa. It appears that messages containing the entire open reading frame lead directly to the production of two forms of the same protein. It is reasonable to propose that the two forms arise from translation that is initiated at different points in the open reading frame of the mRNA.

Results of this study indicate that Sec18p represents a relatively minor fraction total protein production in yeast cells. From quantitation of the relative levels of production of Sec18p and CPY by densitometry, we estimate that Sec18p is produced at only 1/5th to 1/10th of the level of CPY. Twentyfold overproduction of Sec18p was observed in cells harboring the *SEC18* gene on a multicopy plasmid (pSEY8). This suggests that *Sec18* is expressed constitutively and is subject to simple gene dosage control. Experiments looking at the production of Sec18p from the mutant *sec18-1*(Ts) allele show that the temperature-sensitive defect does not result from a decrease in Sec18p expression. In addition, the temperature-sensitive forms of the protein appear to show the same stability at the nonpermissive temperature as wild-type proteins do in pulse-chase labeling experiments (data not shown).

Intracellular location of Sec18p. Our data predict that Sec18p is probably a cytoplasmic protein. To determine this directly, we used cell fractionation techniques with differential centrifugations to separate yeast cells into membrane-enclosed and cytoplasmic compartments. Because of its role in transport between the ER and Golgi complex, we were especially interested to see whether Sec18p might be associated with these compartments. A procedure giving gentle osmotic lysis was developed which appeared to maintain the integrity of small organelles such as mitochondria, ER, and the Golgi complex. Cells were spheroplasted extensively before they were labeled, and then they were washed and labeled with Trans^{35}S label in the presence of full osmotic support (1.2 M sorbitol). To lyse the cells osmotically, cells were pelleted gently, suspended in a small volume of buffer with full osmotic support, and then quickly diluted in a buffer with partial osmotic support (0.25 M sucrose). After an initial low-speed spin to pellet any unlysed cells, the cleared lysate was centrifuged sequentially in a microfuge (13,000 × *g* for 15 min) and a ultracentrifuge (100,000 × *g* for 30 min).

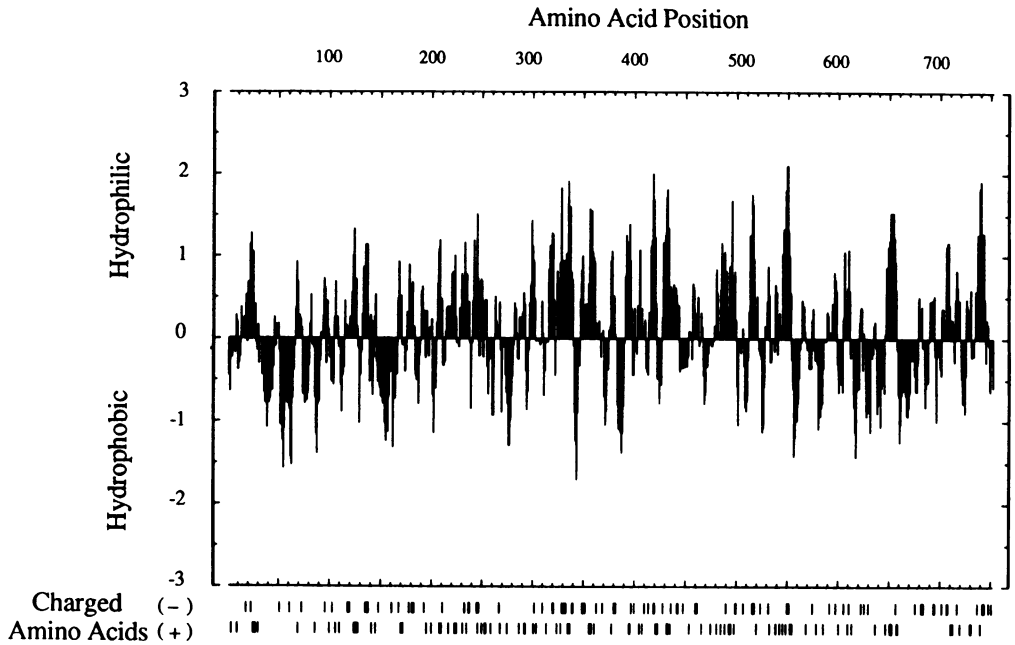

FIG. 7. Hydrophobicity analysis of Sec18p. A hydrophobicity profile of the predicted protein sequence of the *SEC18* open reading frame was calculated by using the values of Hopp and Woods (9) and a window of six amino acids. The positions of charged amino acid residues are indicated by the tics at the bottom of the figure. Neither the entire open reading frame nor open reading frames starting at 19 or 21 amino acids into the protein sequence revealed a satisfactory signal sequence or membrane-spanning domain.

Judging from trichloroacetic acid-precipitable counts, only 30 to 50% of cells were lysed by this procedure. However, no enhancement of any particular form of the markers used was observed, which suggests that the cleared lysate is representative of the contents of the cell.

Fractions were immunoprecipitated with Sec18p antisera under conditions of antibody excess to ensure that comparisons of the level of Sec18p present in the various fractions were valid. Likewise, intermediates in the maturation of CPY were monitored by using CPY-specific antisera to give markers for the ER, Golgi complex, and vacuolar compartments (28). Immunoprecipitation with antisera against the β-subunit of mitochondrial F_1-ATPase was also done to assess the fractionation and intactness of mitochondria in this procedure. Figure 9 shows an autoradiograph of immunoprecipitates of CPY and Sec18p from such an experiment, which is quantitated in Table 1. The p1 and p2 forms of CPY, which should be localized within the ER and Golgi complex, respectively, were mainly present (80%) in the 13,000 × *g* pellet. A small amount of p1 and p2 (20%) was found in the 100,000 × *g* pellet fraction, perhaps because of some fragmentation of these compartments or the material present in small vesicles in transit between secretory organelles. Greater than 95% of the β-subunit of mitochondrial F_1-ATPase was also pelleted in a microfuge. These results indicate that organelles such as ER, Golgi complex, and

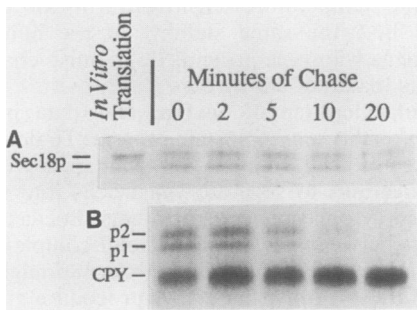

FIG. 8. Pulse-chase labeling of Sec18p and CPY. Yeast cells (strain SEY2101; 1 OD_{600} unit per lane) were labeled for 30 min with $^{35}SO_4$ at 30°C and chased for the indicated times following the addition of $(NH_4)_2SO_4$ to a final concentration of 50 mM. Labeling was stopped by the addition of trichloroacetic acid to 5%, and cell extracts were immunoprecipitated simultaneously with saturating amounts of both Sec18p and CPY antisera. (A) Two forms of Sec18p were not posttranslationally modified into a single species; (B) p1 (ER) and p2(Golgi complex) forms of CPY were chased posttranslationally into the mature vacuolar form. In panel A exposure of the gel was 2 times longer than that in panel B. From densitometry of the bands, we estimate that Sec18 is produced at approximately 1/10th the level of CPY.

TABLE 1. Quantitation of cell centrifugation[a]

Fraction	% of fraction in[b]:		
	Pellet from:		Supernatant from 100,000 × *g* centrifugation
	13,000 × *g* centrifugation	100,000 × *g* centrifugation	
Sec18p	<1	44	56
p1 and p2 CPY	80	20	<1
Mature CPY	<5	<1	>95
Mitochondrial F_1-ATPase β	>95	<1	<5

[a] Quantitation was done by scanning autoradiographs on a laser densitometer (2202; LKB Instruments, Inc., Rockville, Md.) and integration with a program (GelScan; LKB).

[b] Percentages represent the amount present in a given fraction divided by the total of all three fractions. In all cases, the quantitative sum of all three fractions was within 10% of the quantitation of a similar whole-cell lysate sample. Samples which were <5 or <1% were either too faint for accurate quantitation or difficult to resolve because of the presence of contaminating background bands.

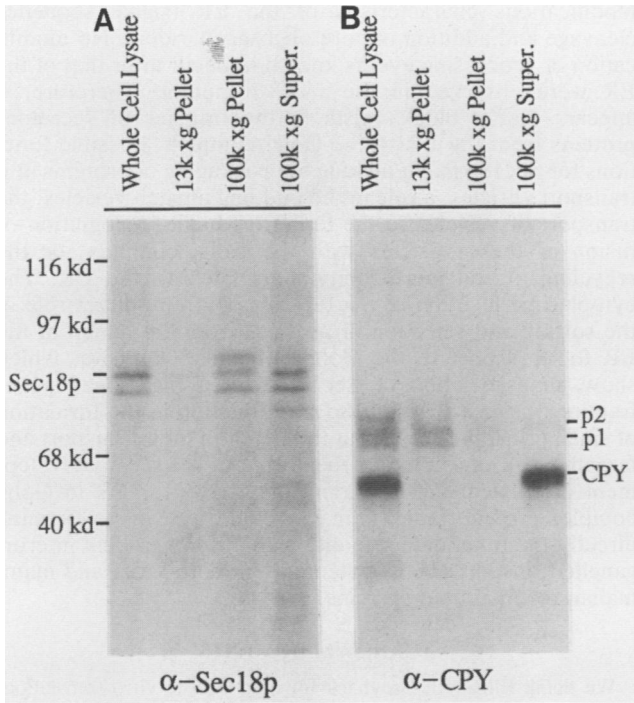

FIG. 9. Sec18p fractionates between a high-speed membrane and supernatant fractions. Yeast cells (strain SEY2101) were spheroplasted with oxylyticase, labeled with Trans^{35}S label, and lysed osmotically. The lysate was pelleted at 2,000 × *g* to remove any unbroken cells, and the supernatant was pelleted sequentially at 13,000 × *g* and 100,000 × *g*. From left to right, the first lane of each panel shows immunoprecipitation from a fraction of the whole-cell lysate, the second lane from a 13,000 × *g* pellet, the third lane from a 100,000 × *g* pellet, and the fourth lane from trichloroacetic acid-precipitated proteins from the 100,000 × *g* supernatant. (A) Immunoprecipitation with Sec18p antiserum; (B) subsequent immunoprecipitation with CPY antiserum. p1 and p2 represent the ER and Golgi complex intermediates, respectively, in the processing of CPY to its mature form. Extra bands that were present in the lane with the Sec18p 100,000 × *g* pellet and the lane with the CPY whole-cell lysate were not reproducible and probably represented some nonspecific cross-reaction of the antiserum that was used for immunoprecipitation. kd, Kilodaltons.

mitochondria appear to remain largely intact by this lysis procedure and are recovered predominantly in the 13,000 × *g* pellet. In contrast, lysis of whole cells by a more vigorous procedure (vortexing in the presence of glass beads) led to 60 to 80% release of the ER, Golgi complex, and mitochondrial markers into the supernatant fraction (data not shown). Very little (<5%) of the mature CPY, a protease that is present in the lumen of the yeast vacuole, was associated with the 13,000 × *g* pellet fraction. The yeast vacuole is a very fragile compartment which we would expect to be lysed without full osmotic support. This was confirmed by the presence of mature CPY predominantly (>95%) in the 100,000 × *g* supernatant fraction.

Sec18p was found in significant amounts in both the 100,000 × *g* pellet (44%) and supernatant (56%) fractions. Little (<1%), if any, of Sec18p was associated with the microfuge pellet fraction. Since Sec18 is implicated in transport between the ER and the Golgi complex, it is somewhat surprising that it would be found in a high-speed pellet fraction in which small vesicles would presumably be recovered and yet not be associated with a fraction (the 13,000 × *g* pellet) in which the bulk of the ER and the Golgi complex were represented. The fact that mature CPY was not associated with the high-speed pellet fraction indicates that the Sec18p found in the pellet was not the result of nonspecific trapping. The data suggest that Sec18p does not interact directly with ER or the Golgi complex within the cell, or does so only very transiently. The presence of significant amounts of Sec18p in the 100,000 × *g* pellet fraction, along with a small proportion of the ER and Golgi complex forms of CPY, suggests that Sec18p may associate with small vesicles in transit from the ER to the Golgi complex. It is also possible that Sec18p binds to some large macromolecular complex such as the cytoskeletal network which could be pelleted at the higher *g* force. The possibility remains that the association of Sec18p with the 100,000 × *g* pellet may be an artifact caused by aggregation of the protein or binding to membrane fragments. Nevertheless, the association of Sec18p with the high-speed pellet may provide a clue to the functional role of Sec18 within the cell. Note also that there was not a preferential association of one or the other form of Sec18p with the pellet fraction, which suggests that the two protein forms may be functionally equivalent.

DISCUSSION

As a first step in understanding the role of the yeast *SEC18* gene product in secretory protein transport between the ER and the Golgi complex, we cloned and sequenced the gene. A single 3.0-kb DNA fragment was isolated that complemented the temperature-sensitive defect exhibited by *sec18-1* mutant yeast cells. This DNA segment mapped genetically to the *SEC18* locus. RNA probes derived from the cloned segment detected one major 2,500-nucleotide poly(A)$^+$ mRNA on Northern blots (Fig. 4A). Consistent with this, the major open reading frame (2,271 bp) in the *SEC18* clone was predicted to encode a protein of 84 kilodaltons. Somewhat surprisingly, two protein products of this open reading frame were observed both from in vitro translation extracts programmed with *SEC18* mRNA and from in vivo ^{35}S-labeled yeast cell extracts (Fig. 5). Disruption of this open reading frame indicates that the function of Sec18p is essential for cell viability. Spores containing a disrupted *SEC18* gene were able to germinate but only underwent, at most, one round of cell division in the absence of *SEC18* expression.

Several aspects of *SEC18* gene expression were novel and unexpected. First, sequences upstream from the site of transcription initiation did not appear to be necessary for *SEC18* expression. A subclone which contained only five nucleotides preceding the start point of transcription was sufficient for the expression of adequate levels of Sec18p for the complementation of the *sec18-1* mutation. Complementation with this subclone was observed when several different high- and low-copy-number yeast shuttle vectors were used, suggesting that aberrant expression of this subclone in a particular plasmid context is not the cause of the observed complementation.

Second, there were two sequences just upstream of the open reading frame, TATATT at bases 516 to 522 and TATAAA at 529 to 535, which could possibly function as TATA elements for transcriptional control. Yet, these sequences were actually within the transcribed region itself rather than at the normal position that TATA elements usually occupy 40 to 120 bp 5′ of the site of transcription initiation (29). This, along with the fact that the transcribed region alone appeared to be sufficient for gene expression, raises the possibility that *SEC18* promoter elements may function from within the gene itself. Alternatively, these

TATA elements formed part of a cryptic promoter leading to expression of the minimum complementing fragment.

Third, the *SEC18* gene produced two proteins from transcripts that appeared to start at a single site. Other cases of the expression of multiple forms of protein from a single yeast gene have been observed, such as the production of cytoplasmic and secreted forms of invertase from the yeast *SUC2* gene (2). However, these were caused by multiple sites of transcription initiation leading to messages which had different AUG codons of the open reading frame at their 5′ ends. There is a precedent for translation initiating at multiple AUG codons in a yeast mRNA. The *GCN4* gene of *S. cerevisiae*, whose protein product controls the transcription of a large number of amino acid biosynthesis genes, uses translation initiation at multiple AUG codons in the mRNA as a translational control of its expression (17). Four short open reading frames of an AUG codon followed by two to three codons are present in an ~600 nucleotide leader region of the *GCN4* mRNA and act to inhibit the translation of *GCN4* under nonstarvation conditions (17). In contrast, *SEC18* appeared to have only one major class of mRNA with a short 5′-untranslated leader and a set of three AUG codons early in the mRNA that were all in frame with the major open reading frame of the gene, yet two forms of the same protein with different sizes were produced (Fig. 4 and 5). The two forms of the protein did not appear to be the result of posttranslational processing events (Fig. 8). The size of the smaller protein product corresponded well to the predicted size of translation products initiated from AUGs that were 19 or 21 codons downstream of the first AUG codon of the open reading frame, leading us to suggest that the smaller protein product arises from translation initiating at one of these downstream codons. In vitro mutagenesis of the different AUG codons in *SEC18* should reveal whether translation indeed starts at more than one site or whether another mechanism is responsible for the multiple forms of protein being produced. In addition, such in vitro mutagenesis should permit one to test whether the two Sec18 proteins are functionally equivalent. It is tempting to speculate that the two isoforms of Sec18p could have different functional roles in directing secretory protein traffic.

The intracellular location of Sec18p was analyzed to gain insight into the functional role that Sec18 plays in the secretory pathway. The predicted amino acid sequences of Sec18 proteins did not indicate any targeting signal which would allow Sec18p to be translocated across the ER membrane. The conclusion that Sec18p remains in the cytoplasm was reinforced by the observation that Sec18p was not modified with core oligosaccharides (Fig. 5), a function which is carried out within the ER, even though potential sites for N-linked glycosylation are present in the amino acid sequence. Results of cellular fractionation experiments by an osmotic lysis procedure, which was able to maintain the integrity of organelles such as mitochondria, ER, and the Golgi complex, indicated that Sec18p is not tightly associated with the bulk of ER or the Golgi complex (Fig. 9). However, centrifugation at higher *g* forces revealed a distribution of Sec18p between the pellet and soluble fractions. This observation suggests that Sec18p may be associated with small vesicles or a macromolecular complex that is important in intracellular secretory protein transport.

The phenotype of *sec18-1* mutant yeast cells suggests a direct role for Sec18p in the transport of secretory proteins between the ER and the Golgi complex. At the nonpermissive temperature, there was a rapid accumulation of newly synthesized secretory proteins which underwent all of the modifications characteristic of the ER (signal sequence cleavage and addition of core oligosaccharides). No modification or processing events known to occur after that of the ER were observed in the *sec18-1* mutant. Therefore, it appears that a block exists in the transfer of secretory proteins from the ER to the Golgi complex. Possible functions for Sec18p might include the packaging of proteins into transport vesicles, a role in the budding of such vesicles, the transport of vesicles to the target organelle, recognition or fusion of these vesicles with the Golgi complex, or the recycling of transport components back to the ER. The cytoplasmic location of Sec18p rules out any direct role in the sorting and selection of proteins from the lumen of the ER for transport to the Golgi complex. Our data, which show an association of Sec18p with a high-speed pellet fraction, suggest that Sec18p may function in the formation of small transport vesicles at the ER or in their transport and targeting to Golgi complex membranes. The recent development of efficient in vitro transport assays for ER to Golgi complex protein traffic (1) may provide a means to examine directly the functional role of Sec18p in this critical interorganelle transport event, potentially in both yeast and mammalian reconstituted systems.

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