Sec2 Protein Contains a Coiled-Coil Domain Essential for Vesicular Transport and a Dispensable Carboxy Terminal Domain

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Abstract. *SEC2* function is required at the post-Golgi apparatus stage of the yeast secretory pathway. The *SEC2* sequence encodes a protein product of 759 amino acids containing an amino terminal region that is predicted to be in an α-helical, coiled-coil conformation. Two temperature-sensitive alleles, *sec2-41* and *sec2-59*, encode proteins truncated by opal stop codons and are suppressible by an opal tRNA suppressor. Deletion analysis indicates that removal of the carboxy terminal 251 amino acids has no apparent phenotype, while truncation of 368 amino acids causes temperature sensitivity. The amino terminal half of the protein, containing the putative coiled-coil domain, is essential at all temperatures. Sec2 protein is found predominantly in the soluble fraction and displays a native molecular mass of >500 kD. All phenotypes of the temperature-sensitive *sec2* alleles are partially suppressed by duplication of the *SEC4* gene, but the lethality of a *sec2* disruption is not suppressed. The *sec2-41* mutation exhibits synthetic lethality with the same subset of the late acting *sec* mutants as does *sec4-8* and *sec15-1*. The Sec2 protein may function in conjunction with the Sec4 and Sec15 proteins to control vesicular traffic.

TRANSPORT of material from one organelle to another in eukaryotic cells is generally mediated by vesicular traffic (Palade, 1975). The molecular events involved in the formation of vesicles from the donor compartment, their migration through the cytoplasm, and the recognition of and fusion with the target organelle are not yet understood. Both biochemical and genetic analysis have led to the identification of a number of components of the machinery (Novick and Schekman, 1979; Novick et al., 1980; Newman and Ferro-Novick, 1987; Segev et al., 1988; Wattenberg and Rothman, 1986; Block et al., 1988; Weidman et al., 1989). Since all vesicular transport events appear to share the same basic mechanism, some of these components may be required for all vesicular transport events. Such a general role has been suggested for the product of the *SEC18* gene (Wilson et al., 1989). In addition to components that act generally, there must also be components that are required for only a single event or subset of events. Components involved in regulating vesicle target recognition would be necessarily limited in their range of function. Candidates for event-specific elements are the 10 *SEC* gene products that have been implicated in the final stage of the yeast secretory pathway (Novick et al., 1980). Defects in these genes block the pathway at the post-Golgi stage, but do not prevent transport from the endoplasmic reticulum to the Golgi apparatus or transport through the Golgi apparatus.

One of the gene products specific for the post-Golgi apparatus stage is the GTP binding protein Sec4 (Salminen and Novick, 1987). This protein appears to function in a cycle. It is synthesized as a soluble protein but rapidly associates with the cytoplasmic surface of secretory vesicles in a process that requires the carboxy terminal cysteines (Goud et al., 1988; Walworth et al., 1989). The vesicles fuse with the plasma membrane; however, Sec4 can reassociate with a new round of secretory vesicles. This cycle of localization may be obligatorily coupled to a cycle of binding and hydrolysis of GTP (Bourne, 1988; Walworth et al., 1989). Strong genetic interactions have been seen between *SEC4* and several other late acting *SEC* genes, including *SEC2* and *SEC15* (Salminen and Novick, 1987). Recent experiments suggest that Sec15 may function in response to Sec4 and Sec2 to dock secretory vesicles onto the plasma membrane (Salminen and Novick, 1989). In this paper, we report the analysis of the *SEC2* gene and its protein product. We present evidence that the Sec2 protein consists of two domains, an essential amino terminal domain that may contain a coiled-coil region and a dispensable carboxy terminal domain.

Materials and Methods

Yeast Strains and Genetic Techniques

Table I lists the yeast strains used for this study. All yeast cultures were grown either in rich medium (YPD)[1] containing 1% Bacto yeast extract, 2% Bacto peptone (Difco Laboratories Inc., Detroit, MI), and 2% glucose or in minimal medium (SD) containing 0.7% yeast nitrogen base without amino acids (Difco Laboratories Inc.), 2% glucose, and when necessary,

1. *Abbreviations used in this paper*: ORF, open reading frame; SD, minimal medium; YPD, rich medium.

Table I. Relevant Yeast Strains

NY 13	*MAT a, ura3-52*
NY 15	*MAT α, ura3-52, his4-619*
NY 26	*MAT a, ura3-52, sec2-59*
NY 130	*MAT a, ura3-52, sec2-41*
NY 363	*MAT a/α, leu2-3, 112/+, ura3-52/ura3-52, his4-619/+*
NY 510	*MAT α, ura3-52, his4-619, sec2-41, GAL+*
NY 562	*MAT α, ura3-52, his4-619, sec2-41, SEC4::pNB229, (SEC4, URA3)*
NY 567	*MAT a, ura3-52, pNB134 (2u,SEC2,URA3)*
NY 673	*MAT α, ura3-52, his4-619, sec2-70, SEC2::pNB289 (sec2-70,URA3)*
NY 674	*MAT α, his4-619, ura3-52::pNB289 (sec2-70, URA3)*
NY 688	*MAT α, ura3-52, his4-619, sec2-71, SEC2::pNB298 (sec2-71, URA3)*
NY 689	*MAT α, his4-619, ura3-52::pNB298 (sec2-71, URA3)*
NY 692	*MAT a, ura3-52, sec2-59, pNB305 (Sup9e, URA3)*
NY 693	*MAT a, ura3-52, sec2-41, pNB305 (Sup9e, URA3)*
NY 694	*MAT a, ura3-52, sec2-59, pNB306 (SupS1, URA3)*
NY 695	*MAT a, ura3-52, sec 2-41, pNB306 (SupS1, URA3)*
NY 696	*MAT a, ura3-52, sec2-59, pNB307 (SUQ5, URA3)*
NY 697	*MAT a, ura3-52, sec2-41, pNB307 (SUQ5, URA3)*
NY 701	*MAT a/α, leu2-3, 112/+, ura3-52/ura3-52, his4-619/+, SEC2::pNB298 (sec2-71, URA3)*
NY 702	*MAT a/α, leu2-3, 112/+, ura3-52/ura3-52, his4-619/+, ura3-52::pNB298 (sec2-71,URA3)*

supplemented for auxotrophic requirements, as described by Sherman et al. (1974).

All yeast transformations were performed by the alkali cation treatment method (Ito et al., 1983). Transformants were selected on SD medium at 25°C, the only exception being NY15 transformants, for which SD supplemented with histidine was used as the selection medium, at 25°C. To initiate complementation assays, yeast transformants were grown on SD medium at 25°C for 3 d. Colonies were then replica stamped to YPD plates and incubated overnight at 37°C and 25°C, at which time colony growth was evaluated. Transformants exhibiting growth at the restrictive temperature were streaked to single colonies on YPD plates at 37°C, and growth was compared to that of a wild-type strain at the same temperature. Suppression analysis also began with yeast transformants grown 3 d at 25°C on SD medium. Transformants were then stamped onto YPD plates and grown overnight at 30, 36, and 37°C. The next morning, growth at the various temperatures was compared. Recovery of plasmids from yeast transformants has been described earlier (Salminen and Novick, 1987). Genetic crosses, the sporulation of diploids, and dissection of tetrads were according to the methods described by Sherman et al. (1974).

Nucleic Acid Techniques

The *Escherichia coli* strain DH1 (F-, recA1, endA1, gyrA96, thi-1, hsdR17, supE44, redA1, lambda-) was used for all cloning experiments. Plasmid manipulations generally followed procedures outlined by Silhavy et al. (1984).

Plasmid pNB70 was isolated by complementation from a yeast genomic library, described by Rose et al. (1987), generated by the introduction of wild-type yeast inserts into the single-copy shuttle vector YCp50 (yeast CEN4, URA3; amp^R, tet^R). The plasmid pNB132 was constructed by inserting the 4.5 kb Cla I-Bam HI fragment from pNB70 into the Cla I and Bam HI sites of YCp50. Insertion of this same 4.5-kb fragment into YIp5 (Struhl et al., 1979) and the 2u-based vector pRB307 (from the collection of D. Botstein) resulted in the integrating plasmid pNB133 and the high copy plasmid, pNB134 respectively. Construction of pNB313 required the deletion, from pNB132, of the 1.73-kb Xba I-Bam HI fragment, arising from cleavage at the internal Xba I site and the Bam HI site. Under conditions favoring partial Xba I digestion, pNB132 was cut with Xba I and Bam HI, and the resulting mixture was treated with Klenow fragment (Boehringer Mannheim Diagnostics Inc., Houston, TX) to fill in the 3′ recessed ends. Subsequent separation of the fragments on a 0.5% agarose gel permitted isolation of the plasmid lacking the 1.73 kb Xba I-Bam HI fragment by the phenol extraction method described by Benson (1984). Blunt end ligation of the plasmid using T4 DNA Ligase (Boehringer Mannheim Diagnostics Inc.) regenerated the Bam HI site at the former Xba I site.

Plasmid pNB209 contains TrpE fused to an internal *SEC2* fragment (185–1016 bp; amino acids 61–338). To generate pNB209, pATH1 (Dieckman and Tzagaloff, 1985) was first digested with Sac I and the 3′ overhang digested back to a blunt end with Klenow fragment. After phenol extraction, the vector was cut with Bam HI (both Sac I and Bam HI sites are located in the polylinker region of pATH1), electrophoresed on a 0.8% agarose gel, and purified. To obtain the insert, the 1.1-kb Pvu II-Bam HI fragment was isolated from pNB313 and digested with Dra I. The 831-bp Dra I-Bam HI fragment, after purification, was inserted, in frame, into the vector and ligated with T4 DNA ligase, blunt end at the Sac I-Dra I juncture, sticky end at the Bam HI-Bam HI juncture.

For construction of the integrating plasmid pNB289, the 3.3-kb Pvu II-Bam HI fragment from pNB132 was isolated and subsequently digested with Rsa I and Ava II. This mixture was then Klenow treated. The blunt-ended 897-bp Rsa I-Ava II fragment, purified from a 0.8% agarose gel, was ligated in to YIp5 cut with Cla I, treated with Klenow, and dephosphorylated with calf intestinal alkaline phosphatase (Boehringer Mannheim Diagnostics Inc.). Xba I and Bam HI digestion of the construction revealed the insert orientation. Construction of another integrating plasmid, pNB298, involved purification of the 1.97 kb Dra I-Dra I fragment of pNB132 after digestion with Cla I, Bam HI, and Dra I. Sau IIIA digestion of this fragment yielded five subclones, the largest of which, an 865-bp Sau IIIA-Sau IIIA insert, isolated from a 1.0% agarose gel, was ligated into YIp5 cut with Bam HI and treated with calf intestinal phosphatase. Insert orientation was determined by digestion with Eco RI and Xba I.

Two alleles of *SEC2*, *sec2-41* and *sec2-59*, were cloned on the plasmids pNB302 and pNB303, respectively. Plasmid pNB132 was cut to completion with Xba I, these "gapped" molecules were then repaired in vivo by chromosomal copies of the respective mutant yeast strains. Presence of the *sec2* locus on the recovered plasmids was determined by comparison of Pvu II and Xba I restriction patterns with those of pNB132 and subsequently by DNA sequence analysis.

Three suppressor tRNA genes were generously donated by Dr. Ian Willis and Dr. Dieter Söll (the Molecular Biophysics and Biochemistry Department, Yale University). All three plasmids have been renamed for this study: pNB305 contains the opal suppressor sup9e (Willis et al., 1984), pNB306 carries the amber suppressor supS1 (Krupp et al., 1985), and pNB307 carries the ochre suppressor SUQ5 (Olson et al., 1981). YCp50 served as the parent vector for all three constructions.

DNA Sequencing Strategy

Nucleotide sequencing was performed using the dideoxy chain termination method devised by Sanger et al. (1977), in the presence of [α-35S]dATP (650 Ci/mmol; Amersham Corp., Arlington Heights, IL) (Williams et al., 1986). Sequencing reactions were electrophoresed on 8% acrylamide gels (40:1.3% acrylamide/bis) containing 8 M urea. DNA templates for sequencing contain restriction fragments of the 4.5-kb Cla I-Bam HI insert of pNB132 subcloned into the M13 phage derivatives mp18 and mp19 and were used in combination with a [-20] 17-mer Sequencing Primer (No. 1211; New England Biolabs, Beverly, MA) or one of three 17-mer primers

generated to specific regions of the *SEC2* gene. For sequence analysis of the *sec2-59* allele, restriction fragments of pNB303 subcloned into M13mp18 and mp19 served as template DNA.

Protein Sequence Homology and Secondary Structure Analysis

The homology search conducted compared the predicted Sec2 protein with the National Biomedical Research Foundation library using the FASTP program in the ktup-2 mode (Lipman and Pearson, 1985). Secondary structure analysis of the Sec2 protein used the Chou and Fasman parameters (Chou and Fasman, 1978) and was plotted using the PLOTSTRUCTURE program of the University of Wisconsin Genetics Computer Group (UWGCG) package.

Preparation of TrpE-SEC2 Fusion and Rabbit Immunization

Production of fusion protein was induced in the *E. coli* strain, NRB209, a DH1 transformant containing the pNB209 plasmid, as described by Goud et al. (1988). A 20-ml culture was harvested at 4,000 rpm for 10 min, washed in 25 mM Tris (pH 8.0), and resuspended in 1.0 ml of 10 mM NaPi (pH 7.2), 1% 2-mercaptoethanol, 1% SDS, 6 M Urea, and incubated at 37°C for 30 min. The sample was then transferred to a 1.5-ml microfuge tube and spun at 13,000 rpm at 4°C for 20 min to remove nucleic acids. The supernatant was collected in a fresh tube and Bromophenol Blue added to a final concentration of 0.05%. Electrophoresis on a preparative SDS polyacrylamide gel separated the proteins in the whole cell lysate and permitted the excision of the fusion protein band, after staining with Coomassie brilliant blue. The gel band was cut into pieces, destained, soaked in distilled water for 2 h, and stored at −20°C. Fusion protein gel slices homogenized in 0.5 ml of PBS and an equal volume of Freund's Complete Adjuvant was administered to anesthetized rabbits subcutaneously for the primary injection. All subsequent boosts were also subcutaneous, at 1-mo intervals, using fusion gel cubes homogenized in PBS alone. The acrylamide serves as an efficient adjuvant.

For affinity purification of anti-Sec2 antibodies, the TrpE-Sec2 fusion protein was collected from gel slices by electroelution (Hunkapillar et al., 1983) in SDS-PAGE buffer at 100 V overnight (Laemmli, 1970) and precipitated using an acetone-triethylamine-acetic acid-water mixture (Konigsberg and Henderson, 1983). Fusion protein was then covalently bound to glutaraldehyde-activated polyacrylamide beads (Act-Ultrogel ACA 22; LKB Instruments Inc., Gaithersburg, MD) and affinity purification proceeded as described by Goud et al. (1988). For 3 h at room temperature, 5.0 ml of rabbit serum was circulated through the fusion protein column; antibodies were eluted from the column according to Guesdon and Avrameas (1976).

Electrophoresis and Immunoblotting

Protein samples for SDS-PAGE were heated for 5 min at 100°C in sample buffer and run on 1.0-mm-thick 10% gels as described by Laemmli (1970). Transfer of proteins to nitrocellulose (BA 83; 0.22 μm, Schleicher & Schuell Inc., Keene, NH) proceeded at 250 mA at 4°C overnight. Probing of blots with affinity-purified anti-SEC2 antibody and radio-iodinated ^{125}I-protein A (0.5 μCi/ml; 30 mCi/mg, Amersham Corp.) was accomplished using the basic procedures described by Burnette (1981) and Goud et al. (1988). Blots were blocked for 15 min in TBS buffer (10 mM Tris, 150 mM NaCl, pH 7.5) containing 1% gelatin (Eastman Kodak Corp., Rochester, NY) and 0.5% BSA, then for 15 min in the same buffer containing 0.1% Tween-20 (blocking buffer). Into this blocking buffer the anti-Sec2 antibody was diluted 1:200 and incubated with the blots for at least 2 h. After four 10 min washes in TBS-Tween, blots were incubated for 2 h with ^{125}I-protein A in blocking buffer. All incubations occurred at room temperature. Blots were dried after four 10 min washes and autoradiographed for 3–16 h.

Preparation of Protein Samples and Measurement of Protein Concentrations

Yeast cultures were grown in YPD or SD medium to an A_{600} of 0.6. Cells (30 A_{600} U) were pelleted in a clinical centrifuge, washed, and resuspended in 10 mM Tris (pH 7.5), containing a mixture of protease inhibitors and then broken with glass beads by continuous vortexing for 3 min at 4°C. A small aliquot was removed for determination of protein concentration as described by Walworth and Novick (1987). The remaining portion of the whole cell lysate was boiled for 5 min in sample buffer. Cooled to room temperature, the lysates were then transferred to microfuge tubes and spun 1 min; the supernatants were removed to fresh tubes and stored at −20°C.

Measurement of Invertase Secretion

Procedures for determination of invertase secretion have been described previously (Goud et al., 1988). Cells were grown overnight in YPD at 25°C. Approximately 1.5×10^7 cells were sedimented in a clinical centrifuge and resuspended in 2.0 ml of YP + 0.1% glucose. Half of each sample was pelleted immediately, resuspended in 10 mM NaN_3, and stored at 0°C; the other 1.0 ml of cells was incubated at 37°C (Table III) or 34°C (Table IV) for 1 h, then harvested, and resuspended in NaN_3 at 0°C. Spheroplast lysates were prepared by the addition of 0.5 ml of cell suspension to 0.5 ml of a solution containing 2.8 M sorbitol, 50 mM Tris (pH 7.5), 10 mM NaN_3, 50 mM 2-mercaptoethanol, and 0.05 mg/ml zymolyase 100T and subsequently incubated at 37°C for 40 min, after which the resulting spheroplasts were sedimented and lysed in 0.5 ml of 0.1% Triton X-100. Methods of assaying invertase have been described earlier (Goldstein and Lampen, 1975).

Results

Cloning of the SEC2 Gene

The *SEC2* gene was cloned from a plasmid library containing yeast genomic DNA inserts in the YCp50 shuttle vector (Rose et al., 1987). The plasmid pNB70 (Table II, Fig. 1 *A*) was identified by its ability to complement the temperature-dependent lethality of the haploid strain NY130 (*sec2-41, ura3-52*). Initial restriction analysis followed by a series of deletions identified the 4.5-kb Cla I–Bam HI fragment, contained on the plasmid pNB132, as a fully complementing subclone (Fig. 1 *A*). More extensive mapping lead to estimates of the specific location of the complementing region on the Cla I–Bam HI fragment. When the 1.4-kb Cla I–Pvu II fragment was deleted and the resulting construction introduced into NY130, transformants grew at the restrictive temperature (37°C). However, when transformants were streaked to single colonies at 37°C, growth was found to be severely limited (data not shown). The plasmid pNB313, created by deletion of the 2.1-kb segment extending from the middle Xba I site to the Bam HI site and leaving a 2.38-kb Cla I–Bam HI fragment (the Bam HI site was regenerated at the Xba I site), did not complement the *sec2-41* defect. The plasmid pNB316 containing a 3-kb insert consisting of the two Xba I fragments gave complete complementation upon introduction into NY 130 (Fig. 1 *A*). These data suggest that the *SEC2* gene lies between the two outer Xba I sites. The middle Xba I site is most probably located within the coding region of the *SEC2* gene, while the Pvu II site may cut near an end of the gene.

To confirm the identity of the complementing region, it was necessary to prove that this genomic fragment could direct integration of a linked marker gene, *URA3* in this case, into the *SEC2* gene locus. The plasmid pNB133, constructed by insertion of the 4.5-kb Cla I–Bam HI fragment into the YIp5 integrating plasmid, was cut with Xba I and used to transform the wild-type yeast strain NY15 (*ura3-52, his-619*) to Ura$^+$. A gene duplication results by homologous integration at the locus corresponding to that of the insert, with the *URA3* marker between the two copies (Fig. 1 *C, top line*) (Orr-Weaver et al., 1981). The resulting strain, carrying a duplication of the insert gene, was crossed to NY130 (*ura3-52, sec2-41*). When tetrads were analyzed, tight linkage of

Table II. Relevant Plasmids

pNB70	YCp50, *SEC2*; 11-kb genomic insert in the Bam HI site
pNB132	YCp50, *SEC2*; 4.5-kb Cla I-Bam HI fragment from pNB70 into Cla I-Bam HI sites
pNB133	YIp5, *SEC2*; 4.5-kb Cla I-Bam HI fragment from pNB70 into Cla I-Bam HI sites
pNB134	2μ, *SEC2*; 4.5-kb Cla I-Bam HI fragment from pNB70 into Cla I-Bam HI sites of the vector pRB307
pNB209	pATH1, *TrpE-SEC2* fusion; .84-kb Dra I-Bam HI(Xba I) fragment from pNB313 into Sac I (Klenow-treated) and Bam HI sites of pATH1
pNB289	YIp5, *SEC2*; internal 0.9-kb Rsa I-Ava II fragment (blunt) from pNB132 into the Cla I (blunt) site
pNB298	YIp5, *SEC2*; internal 0.86-kb Sau III A-Sau III A fragment from pNB132 into the Bam HI site
pNB302	YCp50, *sec2-41*; pNB132 was gapped with Xba I and introduced into NY 130, then reisolated after plasmid repair at the *sec2-41* chromosomal locus
pNB303	YCp50, *sec2-59*; pNB132 was gapped with Xba I and introduced into NY 26, then reisolated after plasmid repair at the *sec2-59* chromosomal locus
pNB305	YCp50, *sup9e*; 1.55-kb Cla I-Eco RI fragment from a YRp17 clone (opal, *sup9e*) into Cla I-Eco RI sites
pNB306	YCp50, *supS1*; .44-kb Hin D III-Bam HI fragment from YRp51 clone (amber, *supS1*) into Hin D III-Bam HI sites
pNB307	YCp50, *SUQ5*; 1.1-kb Hin D III-Bam HI fragment (ochre, *SUQ5*) into Hin D III-Bam HI sites
pNB313	YCp50, *SEC2*; 2.28-kb Cla I-Bam HI fragment, Xba I (internal)-Bam HI of pNB132, Bam HI site regenerated at Xba I site
pNB316	YCp50, *SEC2*; 3.0-kb Xba I-Xba I fragment cloned into Xba I site of pUC19 and from there into the Hind III and Bam HI sites of YCp50
pNB318	pBR322, *SEC4, LEU2*; 1.4-kb *SEC4* fragment inserted into the Eco RI and Bam HI sites and the *LEU2* gene inserted into the Sal I site

the site of integration with the *SEC2* locus was established: all tetrads consisted of two Ura$^+$, temperature-resistant spores and two Ura$^-$, temperature-sensitive spores. These data strongly suggest that the cloned sequence contains the *SEC2* gene. Definitive proof was obtained by sequence analysis of the *sec2-59* mutant allele as described below.

SEC2 Encodes a Hydrophilic Protein Containing a Possible Coiled-coil Domain

Based on the localization estimates discussed above, sequence analysis of the 4.5-kb Cla I-Bam HI clone was begun, focusing on the region downstream of the Pvu II site. In total, the 3-kb complementing region between the outer Xba I sites was sequenced, within which a single long open reading frame (ORF) of 2,280 bp was revealed (Figs. 1 *B* and 2). This ORF encodes a putative protein product of 759 amino acids with a predicted molecular mass of 84 kD. The Sec2 protein is quite hydrophilic, 32% of the amino acids being charged, 15% basic and 17% acidic. Based on hydrophobicity analysis, there are no stretches of sufficient length and hydrophobicity to constitute a membrane spanning region.

The Sec2 protein sequence was compared with proteins in the PIR database. The amino terminal 200–300 amino acids of the Sec2 protein display significant similarity to the α-helical, coiled-coil portions of four myosins and to several other cytoskeletal proteins, including a Type II keratin, a troponin, and a tropomyosin, the identity ranging from 20 to 26%. In the case of the cardiac myosin α heavy chain, the 24.6% identity with the Sec2 protein over 167 amino acids involves the S2, rod portion of the cytoskeletal protein (Fig. 3 *A*) (Kavinsky et al., 1984). This domain of myosins serves a vital function in the formation and assembly of thick filaments in skeletal muscle and follows a strict 7-residue amino acid repeat pattern, characteristic of α-helical coiled-coil proteins, in which hydrophobic amino acids are found exclusively at two positions of the α-helix forming a hydrophobic stripe along which two similar chains may interact (Crick, 1952). Secondary structure analysis using methods described by Chou and Fasman (1978) and Garnier et al. (1978) both predict a strong α-helical nature for the first 300 amino acids of Sec2 (not shown). Over the region from residue 50 to 106 the sequence closely matches the heptad repeat rules (Fig. 3 *B*). With only 3 exceptions, positions 3 and 6 are occupied by hydrophobic amino acids. Another common feature of coiled-coil proteins is the presence of frequent salt bridges stabilizing the α helix (Sundaralingam et al., 1987; Marqusee and Baldwin, 1987). In this region of the Sec2 protein, there are 15 predicted salt bridges between adjacent residues of the helix (Fig. 3 *B*). An unusual feature of Sec2 is that position 2 is occupied by acidic residues in 6 out of 8 repeats. The significance of this is unclear. On either side of this eight heptad repeat region lie regions of similar size that fit the heptad repeat rules somewhat more loosely and are shifted in frame with respect to the 50–106 region. In the region from residue 1 to 49 two adjacent positions of the helical wheel are occupied by hydrophobic residues in 10 of 14 cases. While in the region from 107 to 162, two adjacent positions of the helical wheel are occupied by hydrophobic residues in 13 of 16 cases. The carboxy terminal half of the protein does not exhibit any striking homologies; however, it is quite hydrophilic and includes a region (residues 542–650) that is 37% serine or threonine.

Production of Anti-Sec2 Antibodies

We raised, in rabbits, polyclonal antisera against a fusion protein comprised of the TrpE protein and a portion of the *SEC2* gene product (amino acids No. 61–338) (see Materials and Methods). Although the *SEC2* sequence predicts a peptide of 84 kD, on immunoblots (Fig. 4) the antisera recognizes a band in wild-type (NY15) whole cell lysates that migrates at 105 kD. To verify the identity of the 105-kD immunoreactive band as the *SEC2* gene product, the 4.5 Cla I-Bam HI fragment was inserted into a high copy number, 2μ-based vector and the resulting plasmid, pNB134, was introduced into a wild-type strain. Transformants were selected on minimal media (SD). On immunoblots, whole cell lysates

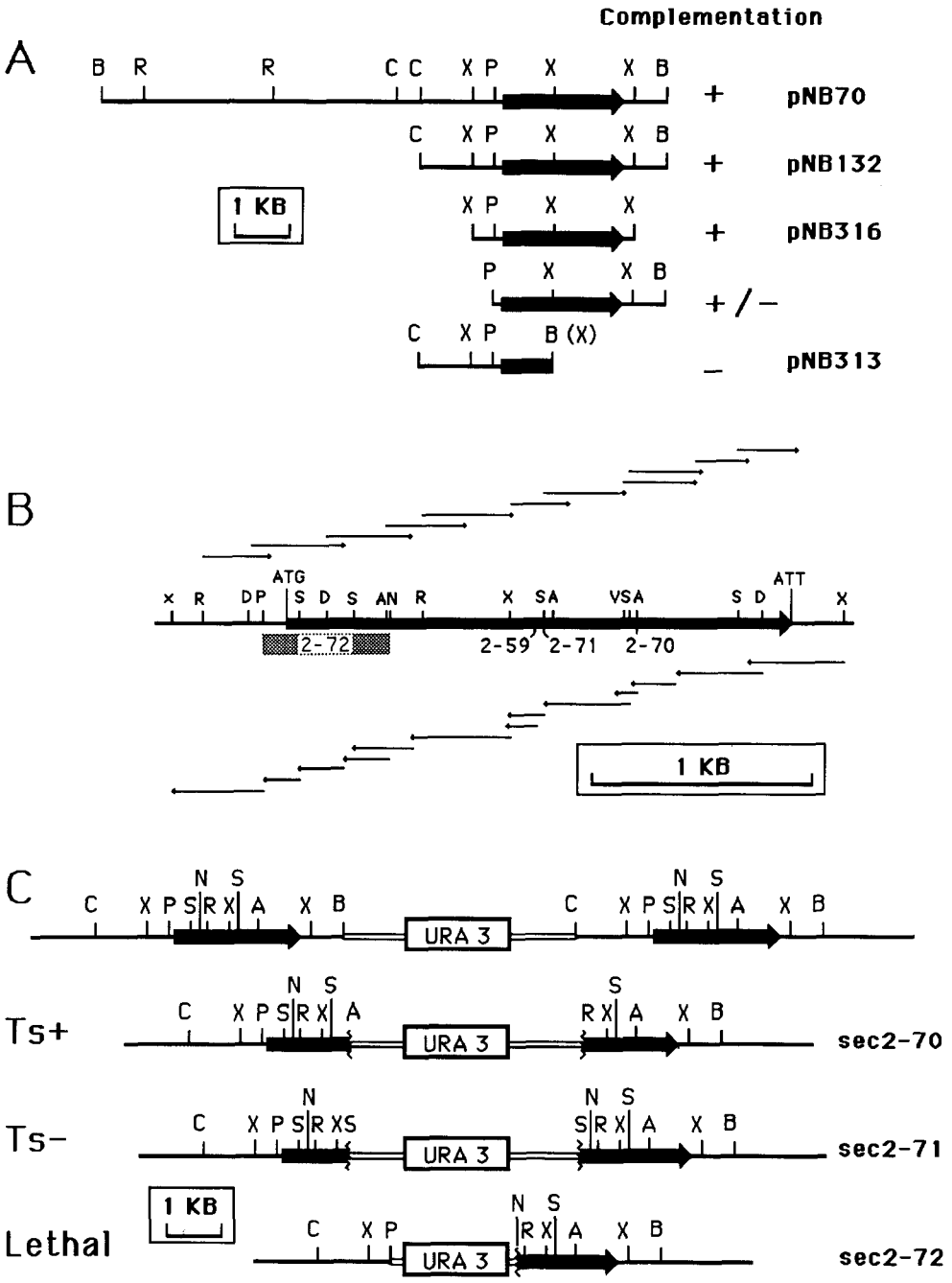

Figure 1. Genetic analysis of the *SEC2* locus. (*A*) Complementation of *sec2-41* by the various subclones described in the text and in Table II. ORF, heavy arrows. (*B*) Restriction map and sequencing strategy of *SEC2*. The start and stop codons are indicated as are various mutations. (*C*) Various alleles generated by integration. The top line shows the integration of pNB133 into the genome, generating a duplication. The *sec2-70* allele was constructed by integration of PNB289 into the genome. The *sec2-71* allele was generated by integration of pNB298. This allele yields a temperature-sensitive phenotype. The *sec2-72* allele was constructed by replacement of the Pvu II–Nsi I region of *SEC2* with the *URA3* gene. *A*, AluI; *B*, Bam HI; *C*, Cla I; *D*, Dra I; *N*, Nsi I; *P*, Pvu II; *R*, Rsa I; *S*, Sau IIIa; *X*, Xba I.

of this overproducer (NY567) show an ~10-fold increase in levels of the same 105-kD band seen in wild-type lysates. The 105-kD band is absent in lysates of two temperature-sensitive *sec2* mutant strains, *sec2-41* and *sec2-59* (Fig. 4); however, new bands appear at much lower apparent molecular mass: a 43-kD band in NY130 (*sec2-41, ura3-52*) and a 41-kD band in NY26 (*sec2-59, ura3-52*). Together these data establish the 105-kD protein as the normal *SEC2* gene product. The surprising finding was the greatly increased mobility of the *Sec2* protein encoded by the temperature-sensitive alleles.

Additional bands observed in wild-type lysates and overproduced in NY567, but not seen in the mutants are probably Sec2 degradation products. Low molecular mass bands, below those of the mutants, are present at equal levels in all samples regardless of variations in Sec2 protein content and, therefore, most likely represent nonspecific binding of the antibody.

An Opal Suppressor tRNA Suppresses the sec2-41 and sec2-59 Defects

There are several possible explanations for the greatly reduced molecular mass of the mutant proteins. The *sec2-41* and *sec2-59* alleles could encode full length, missense proteins that are sensitive to proteolytic degradation either in

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-513  TCTAGAAAAATTGAGGGCCCGCAAGGAAAGATCCATAGGTGAGGCTAGCACAGGTAACAGGCTAAGTTTCAAATAAAATATTAAACCAAAAAATTATGACAACAGTAATACCAAAACAAA
-393  CATCCAAACAAGCACTAGTACAAAGAAATAGTCACGAATCATTAAATCACATATATATATATAATTTTAATGTTTATAGATCACTTATGATACACTTTACATTCATCTTTACTCAAATGA
-273  TCTCTACTATTGCATTTTTGAAATAATTCCTTTTATTATGTGCCATTGTTTCCATGTGACTACCCGGAACTTGTATTTCGCGTTCGCTGAAGCACCTTTAAAACATGGCTCTCGTTTACC
-153  TAAGTGACAAGCACAGTGATTCGTGCAATAATATCATTTGTCACTTGCAGCTGTAGCAACGTTGATTTCCATCCAAAAGTTGTAGAAAATTTTGACCTTTAGTTCACTGGGATATATTTT
                                                    MetAspAlaSerGluGluAlaLysArgValSerIleGlnValThrSerLeuSerThrGlnLeuIleGluSerValAspLysGlnSer
   33  CCTTTACTAGCTGTGTTTGATAAAGGTTTCAATATGGATGCTTCTGAGGAAGCAAAAAGAGTATCAATACAGGTTACATCCCTGTCTACGCAATTGATCGAGAGCGTGGATAAACAGTCA

       HisLeuGluGluGlnLeuAsnLysSerLeuLysThrIleAlaSerGlnLysAlaAlaIleGluAsnTyrAsnGlnLeuLysGluAspTyrAsnThrLeuLysArgGluLeuSerAspArg
   88  CACTTAGAAGAACAGCTTAATAAATCCTTAAAAACAATAGCCAGCCAGAAGCAGCAATTGAAAACTATAATCAGTTGAAAGAAGATTACAACACTTTAAAAAGAGAATTGTCAGACAGA

       AspAspGluValLysArgLeuArgGluAspIleAlaLysGluAsnGluLeuArgThrLysAlaGluGluGluAlaAspLysLeuAsnLysGluValGluAspLeuThrAlaSerLeuPhe
  208  GATGACGAAGTGAAAAGATTGCGTGAAGATATAGCCAAAGAAAATGAGCTTCGAACTAAAGCTGAAGAGGAAGCGGATAAACTAAATAAAGAAGTTGAGGATCTGACTGCTTCCCTCTTT

       AspGluAlaAsnAsnMetValAlaAspAlaArgLysGluLysTyrAlaIleGluIleLeuAsnLysArgLeuThrGluGlnLeuArgGluLysAspThrLeuLeuAspThrLeuThrLeu
  328  GATGAGGCTAATAATATGGTTGCTGATGCTAGAAAGGAGAAATATGCTATCGAAATCTTAAATAAGAGACTCACTGAACAACTGCGTGAAAAGGACACATTACTGGATACTTTGACCCTA

       GlnLeuLysAsnLeuLysLysValMetHisSerLeuAspAsnGluSerThrValThrAsnAsnSerAsnArgTyrSerThrIleLeuSerAspSerAlaThrSerSerSerThrSerLeu
  448  CAGCTCAAGAATCTGAAAAAAGTGATGCATAGTTTAGATAATGAAAGCACAGTCACAAATAATTCGAACAGATATTCCACAATATTGAGTGATTCAGCAACTTCTTCTAGCACTTCTTTG

       AsnLysValProThrSerTyrSerLeuAlaSerGlnAspIleTyrSerGlyIleValTyrSerProSerIleSerSerIleArgTyrAspIleSerLeuTyrAsnGluPheLeuLysPhe
  568  AATAAAGTTCCAACTTCATATTCTCTTGCTTCCCAAGATATTTATAGCGGAATTGTGTACTCGCCAAGCATTTCATCTATTCGTTATGATATAAGCCTTTACAATGAATTTCTAAAATTT

       ValAlaAlaLeuProArgCysGluAsnIleLysAlaThrSerThrGluSerLysLeuIleArgArgLeuValAsnAspGluIleGlnProIleLeuLysIleAspAsnAlaSerGlyIle
  688  GTCGCTGCACTACCCCGTTGTGAAAACATAAAGGCAACTTCAACCGAATCAAAGTTAATACGAAGATTGGTAAATGATGAAATCCAGCCTATACTTAAGATTGATAATGCTTCAGGCATC

       GlyTrpLeuValLysLysThrLeuLeuSerLeuIleIleAspGlyLeuValValValGluProLeuSerGlyValAsnAlaThrTyrGlnIleGlyTyrAsnSerSerSerProAlaLys
  808  GGCTGGTTGGTTAAAAAAACCCTACTTTCCTTGATTATAGATGGTTTGGTGGTCGTGGAACCCCTAAGCGGCGTTAATGCAACCTACCAAATTGGCTACAACTCAAGTAGCCCTGCAAAG

       GlnAlaThrSerAsnMetProLysMetPheLysPheProLeuAspSerProProValAlaValHisAlaAlaCysSerPheCysGlyGluSerArgAspAspIleIleGluHisAlaArg
  928  CAAGCCACTTCGAATATGCCAAAAATGTTCAAGTTTCCCTTAGATTCACCTCCAGTTGCAGTGCACGCCGCATGTTCCTTTTGTGGAGAATCTAGAGATGACATTATCGAACACGCAAGG

       MetTyrIleLeuLysThrLeuHisLysThrAspAspGlyLysGluGlnValThrAsnThrTyrProLeuCysHisTrpCysLeuLeuLysLeuArgGlnThrCysGluIlePheAlaPhe
 1048  ATGTATATATTGAAAACGTTGCACAAGACTGACGACGGTAAAGAACAAGTTACGAATACGTATCCCTTGTGTCATTGGTGTTTGCTAAAGTTGCGGCAGACATGTGAAATCTTTGCTTTC

       LeuArgSerLeuLysValGlyAlaTrpHisLeuGluLysLeuThrThrGlnAsnIleThrLysGluAspLeuGluLysPheSerGluValThrLysHisThrLysArgAspGlyArgVal
 1168  CTGAGATCATTGAAGGTAGGCGCATGGCATTTAGAAAAGCTAACGACGCAGAACATTACCAAGGAAGATTTGGAAAAATTCTCTGAAGTAACGAAGCATACCAAAAGAGACGGCAGGGTT

       SerSerGlnAspLysLysThrLysArgLeuSerPheMetAlaGlyLeuGlyIleAsnSerSerThrLysAsnLysProLysMetGluIlePheSerSerGluThrAsnAlaLysProGly
 1288  TCTTCTCAAGACAAAAAAACCAAACGTTTAAGTTTTATGGCAGGACTTGGTATCAATTCTTCAACAAAAAATAAGCCAAAGATGGAGATTTTTTCTTCTGAAACAAATGCAAAGCCAGGA

       GlnProThrThrAsnIleGlnArgAlaTrpLeuGlnLeuCysLysLeuArgCysIleLeuHisTrpThrHisIleGlyIleTrpAlaValAspAspSerIleSerSerLysIleGlyPro
 1408  CAACCTACCACTAATATTCAAAGAGCATGGCTGCAATTGTGTAAATTGCGTTGTATACTTCATTGGACTCATATTGGCATATGGGCAGTGGATGATTCAATTTCTTCAAAAATTGGACCG

       LeuValGluAspAspSerAspGluAspGlnAsnAspAlaIleSerValArgLeuGlnAspLysAlaLeuTrpLysGlnAspAlaLysArgProPheSerSerSerSerAlaGluGluSer
 1528  TTAGTTGAGGATGACAGCGATGAGGATCAAAACGATGCTATATCGGTTCGCTTGCAGGACAAAGCTCTTTGGAAGCAGGACGCAAAAAGACCTTTTTCTTCATCTAGCGCAGAAGAAAGT

       GlnLysSerAspAlaPheAspPheGluSerGlyAspMetGluAsnGluIleThrGlyGluSerSerSerAspGluSerSerSerAspGlySerSerThrAspAsnSerThrAlaAspSer
 1648  CAAAAGAGCGACGCTTTTGACTTTGAAAGTGGAGATATGGAAAATGAAATTACGGGTGAATCTTCAAGTGATGAATCCTCTTCAGATGGGTCTTCCACAGATAATTCAACCGCAGATAGT

       SerSerGluAspGluSerSerLeuAlaAspSerThrThrSerSerAlaAspSerSerSerProGluSerIleAspAsnGlyGluGlyAspAspThrValThrLysAspAspLysSerSer
 1768  TCATCAGAAGATGAATCTTCTCTAGCAGATTCTACTACAAGTAGCGCAGATTCTAGTTCACCAGAGAGTATAGACAACGGTGAAGGTGATGACACGGTAACGAAAGATGACAAAAGTAGC

       IleLysSerAlaAsnAsnAsnGluGluAsnSerAspCysGlyAspLysLysGlyArgSerIleIleLysLysLysAlaProGlnArgLysIleGlnLysLysLysLeuLeuGlnAspLeu
 1888  ATTAAGTCGGCAAATAATAATGAAGAAAACAGCGATTGCGGAGATAAAAAAGGTCGTTCTATAATAAAGAAAAAGGCTCCTCAACGCAAGATACAAAAGAAGAAGTTACTCCAAGATTTG

       AspAspLeuGluGluGlnPheArgGluGluSerAlaIleAspGlnThrGluPheGluAsnAlaGluSerAsnValLysGlnAsnIleSerSerLysArgAlaSerSerGlyAspGluAsn
 2008  GATGATTTAGAAGAGCAATTTAGAGAAGAAAGCGCAATTGATCAGACTGAATTTGAAAACGCTGAGTCTAATGTAAAACAAAATATATCCTCGAAGAGGGCATCTTCTGGAGACGAGAAC

       SerLysLysAspAsnAsnGluLysThrLeuLysThrAsnLeuThrIleGlyAspLysThrGlnGluGlnIleGlyGluAsnSerProSerSerGlyLeuHisAlaSerSerSerAsnAsp
 2128  TCTAAGAAGGATAATAATGAAAAAACTTTAAAGACAAACTTGACGATAGGCGACAAAACCCAAGAACAAATAGGGGAAAATAGTCCTAGTAGCGGATTGCATGCGTCTTCAAGCAATGAT

       AspAsnPheAspAspAlaGlnGluGlnGln###
 2248  GACAATTTCGATGATGCCCAGGAACAGCAATAAATTATATAATATTTAATTTTATGTGCCTAATGGACATTTGAGTTCCTTAAGATGTTCAACAACTTAGAAAAATATAGGTAATGATAA
 2368  AGAATGAATCGCATGTATTGTTTTAATATTATGTCAATACTTTGAACTTAAAAACAGAGTAAAGAAGTGGTTCTAAAATTACACGTATTAAAGGGATTAATTACTACATATTCATTCTCA
 2488  ATCATCACTATCACCTTGGCTCAAAACAACTCT  2520
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Figure 2. The sequence of *SEC2*. These sequence data are available from EMBL/GenBank/DDBJ under accession number x52147.

vivo or during preparation of the lysates, leading to complete conversion of the 105-kD protein to the shortened forms. The mutant alleles could alternatively encode proteins truncated by deletion or by nonsense codons. If the reduced molecular mass is the result of a premature stop codon, then introduction of an appropriate nonsense codon suppressor tRNA should alleviate the temperatures-sensitive growth defects of these mutants by facilitating the production of full length Sec2 protein. Dr. Ian Willis and Dr. Dieter Söll (the Molecular Biophysics and Biochemistry department of Yale

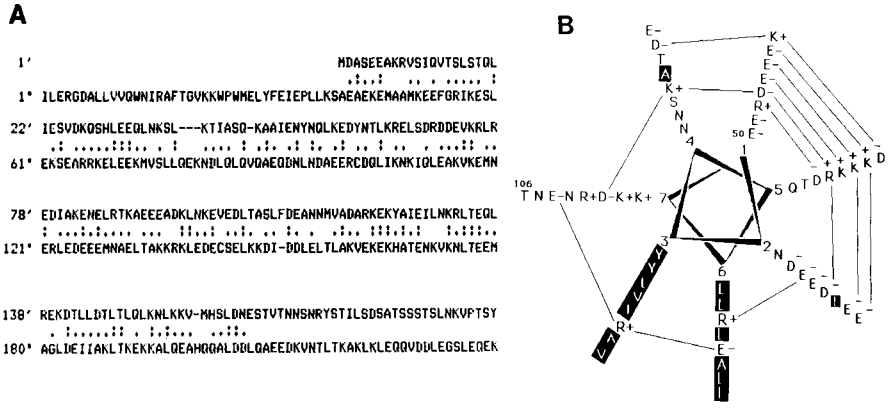

Figure 3. (*A*) Comparison of Sec2 protein with S2 region of cardiac muscle myosin α heavy chain. The top line shows the sequence of the amino terminus of Sec2. The line below is a portion of the S2 region of rabbit cardiac muscle α-myosin heavy chain as reported by Kavinsky et al., 1984. Double dots indicate identity; single dots indicate a conserved change. This comparison represents the optimized lineup as performed by the program of Lipman and Pearson, 1985. (*B*) Helical wheel portrayal of the region of Sec2 from amino acids 50–106. The hydrophobic amino acids are shadowed and the charged amino acids are indicated. Possible salt bridges are shown by the light lines.

University) generously donated three plasmids, renamed pNB305, pNB306, and pNB307, each containing a gene encoding a suppressor tRNA for one of the three stop codons: opal (UGA), amber (UAG), and ochre (UAA). The three genes sup9e (opal), supS1 (amber), and SUQ5 (ochre), from either *Saccharomyces pombe* or *Saccharomyces cerevisiae*, were placed into the YCp50 shuttle vector. NY26 (*sec2-59*, *ura3-52*) and NY130 (*sec2-41*, *ura3-52*) strains were transformed, separately, with each of the three plasmids, and transformants were selected on SD medium at 25°C. After 3 d, transformants were stamped to YPD plates and incubated at 30°C, 36°C, and 37°C overnight. Of the six new strains (NY692-NY697), only the two carrying the sup9e opal suppressor gene, NY692 (*sec2-59*) and NY693 (*sec2-41*), grew at the restrictive temperatures (data not shown). The sup9e gene more effectively suppressed *sec2-41* than *sec2-59*. Suppression of *sec2-41* by sup9e was equally strong at all three temperatures whereas suppression of *sec2-59* was strong at 30°C but weak at 37°C. Immunoblots reveal the production of a small amount of the full length, 105-kD Sec2 protein in the sup9e containing strains but not in those carrying the supS1 and SUQ5 genes (Fig. 5). In addition, more 105-kD protein is present in the *sec2-41* strain (NY693), which is well suppressed than in the *sec2-59* strain (NY692), which is suppressed more weakly.

The efficiency of suppression was determined by a quantitative evaluation of invertase secretion in the mutant strains

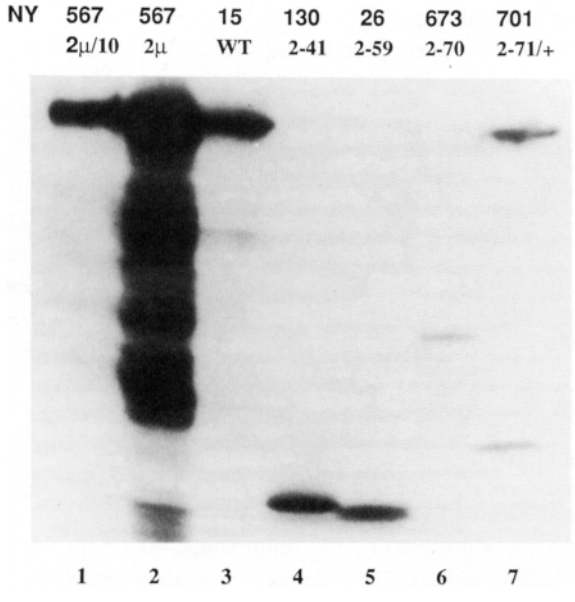

Figure 4. Anti-Sec2 immunoblot analysis of various *sec2* alleles. (Lanes *1* and *2*) NY 567, a strain carrying the *SEC2* gene on a multicopy plasmid. Lane *1* was loaded with one-tenth as much protein as the other lanes on the gel. (Lane *3*) NY 15, a wild-type strain; (lane *4*) NY130, a *sec2-41* strain; (lane *5*) NY26, a *sec-59* strain; (lane *6*) NY673, a *sec2-70* strain; (lane *7*) NY701, a diploid strain carrying one wild-type *SEC2* allele and one *sec2-71* allele.

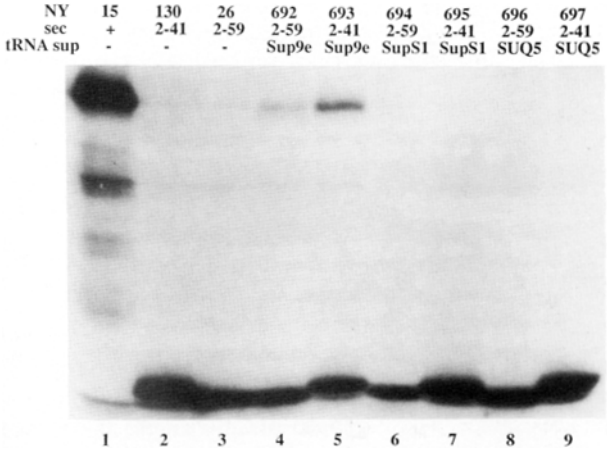

Figure 5. Anti-Sec2 immunoblot of *sec2-41* and *sec2-59* strains carrying tRNA suppressors. (Lane *1*) Ny15, a wild-type strain; (lane *2*) NY130, a *sec2-41* strain; (lane *3*) NY26, a *sec2-59* strain; (lane *4*) NY692, a *sec2-59* strain containing the Sup9e tRNA suppressor; (lane *5*) NY693, a *sec2-41* strain containing the Sup9e suppressor; (lane *6*) NY694, a *sec2-59* strain containing the SupS1 suppressor; (lane *7*) a *sec2-41* strain containing the SupS1 suppressor; (lane *8*) NY696, a *sec2-59* strain containing the SUQ5 suppressor; (lane *9*) NY697, a *sec2-41* strain containing the SUQ5 suppressor.

Table III. Suppression of sec2 Secretion Defect by tRNA Suppressors at 37°C

Strain	*sec*	Suppressor	Invertase Activity (μM glucose/min·A_{600}U) External 0 h	External 1 h	Internal 0 h	Internal 1 h	Invertase secretion (%)
NY 13	*SEC2*	–	.013	.250	.044	.050	98
NY 26	*sec2-59*	–	.014	.024	.033	.624	2
NY 130	*sec2-41*	–	.016	.036	.033	.496	4
NY 692	*sec2-59*	sup9-e	.014	.231	.037	.181	60
NY 693	*sec2-41*	sup9-e	.013	.232	.036	.127	71
NY 694	*sec2-59*	supS1	.018	.025	.037	.599	1
NY 695	*sec2-41*	supS1	.015	.031	.037	.498	2
NY 696	*sec2-59*	SUQ5	.015	.018	.035	.550	1
NY 697	*sec2-41*	SUQ5	.015	.028	.036	.461	3
NY 673	*sec2-70*	–	.013	.154	.037	.053	90

carrying the suppressor tRNA plasmids. Cultures of the relevant strains were grown overnight at 25°C in media containing 2% glucose to repress synthesis of invertase. Cells (2.0 A_{600} U) were pelleted, resuspended in low glucose to derepress invertase production, and incubated 1 h at 37°C. Intracellular and external invertase activity were then measured. The percentage of invertase secreted is a measure of the secretion block. As illustrated in Table III, wild-type strains secrete at levels near 100% while the mutant strains, NY26 and NY130, release only a very small amount of the invertase produced. The presence of either amber or ochre suppressors does not affect the levels of invertase secreted from the mutants. However, when the sup9e gene is introduced, secretion of invertase increases dramatically, rising to 60% in the *sec2-59* strain and to 71% in the *sec2-41* strain.

The efficiency of suppression of the vesicle accumulation phenotype of the mutant alleles was evaluated by thin section electron microscopy (Fig. 6, *A–D*). As has been previously demonstrated, *sec2-41* accumulates secretory vesicles at 37°C. The same strain carrying an opal tRNA suppressor (NY693, Fig. 6 *C*) does not accumulate vesicles at this temperature, while a strain carrying an amber tRNA suppressor (NY 695, Fig. 6 *D*) still shows the mutant phenotype.

Sequence of sec2-59

Final proof of the identity of the *sec2-59* allele lies in the nucleotide sequence. To accomplish the sequencing of the mutant, the allele was cloned using a technique described by Saliminen and Novick (1987). The plasmid pNB132 was digested with Xba I, generating linearized molecules "gapped" over the entire *SEC2* coding region but leaving the flanking sequences intact. These gapped plasmids were introduced into the NY26 strain. The chromosomal sequences repair the gapped regions of the plasmid. The plasmid, reisolated from yeast and purified through *E. coli* contains the *sec2-59* gene. Sequence analysis of the *sec2-59* gene, cloned on the plasmid pNB303, confirmed that the mutation is a premature opal stop codon. At 1,125 bp in the coding sequence, a point mutation occurs, a change from a guanine to an adenine residue, and the resulting codon (1,123–1,125 bp) in the *SEC2* ORF is a UGA opal stop codon (Fig. 2). The *sec2-59* sequence predicts a protein of 374 amino acids and an estimated molecular mass of 41.1 kD, consistent with the band observed on immunoblots (Fig. 4).

SEC2 Is an Essential Locus; However, Truncated Sec2 Proteins Are Still Functional

Two models can account for the temperature-sensitivity of the *se2-41* and *sec2-59* strains. Either Sec2 function is only required at elevated temperatures or Sec2 function is required at all temperatures, and the carboxy terminal half of the protein is only necessary at elevated temperatures. To test these possibilities, we constructed an allele, *sec2-72*, which lacked the region immediately upstream of the protein start as well as the amino terminal coding region. This construction replaced the Pvu II–Nsi I region of *SEC2* with the *URA3* gene (Fig. 1 *C*). This allele was introduced into the genome by the one step gene disruption technique (Rothstein, 1983) so as to replace one copy of *SEC2* in a diploid strain, NY363. Three Ura^+ transformants were sporulated and subjected to tetrad analysis at 25°C. All 30 tetrads analyzed yielded two viable and two inviable spores. No viable Ura^+ were obtained. The inviable spores successfully germinated, and gave rise to microcolonies of 4–8 cells before division ceased. Therefore, *SEC2* is required for vegetative growth at 25°C as well as at elevated temperature. This result also implies that the *sec2-41* and *sec2-59* alleles do encode partially functional Sec2 protein despite the truncation of up to 50% of the protein sequence.

To map the regions of the protein necessary for growth at different temperatures two additional alleles of *SEC2* were generated by truncation of the C-terminus. For construction of the first allele, an internal Rsa I–Ava II fragment, extending from 625–1,524 bp of the coding sequence, was inserted into the YIp5 vector to create the plasmid pNB289 (Table II and Fig. 1, *B* and *C*). Transformants were selected on minimal medium at 25°C. Directed integration at the *SEC2* locus of the haploid wild-type strain NY15 (*ura3-52*, *his4-619*) transformed with pNB289 linearized by Xba I digestion creates a duplication in which both copies of *sec2* are truncated, one at the amino terminus, the other at the carboxy terminus (Fig. 1 *C*, line 2). With neither an AUG codon nor a promoter sequence, the amino terminal truncation cannot serve as a template for transcription. Therefore, the only Sec2 protein produced in this transformed strain (NY673) originates from the carboxy terminal truncated gene, designated *sec2-70*, encoding a protein of 519 amino acids, 508 from *SEC2*

Figure 6. Thin section analysis of *sec2-41* with suppressor plasmids. (*A*) NY13, a wild-type strain incubated at 37°C for 1 h. (*B*) NY130, a *sec2-41* strain incubated at 37°C for 1 h. (*C*) NY693, a *sec2-41* strain carrying the Sup9e suppressor incubated at 37°C for 1 h. (*D*) NY695, a *sec2-41* strain carrying the SupS1 suppressor incubated at 37°C for 1 h. (*E*) NY130, a *sec2-41* strain incubated at 34°C for 1 h. (*F*) NY723, a *sec2-41* strain carrying an episomal copy of *SEC4* incubated at 34°C for 1 h.

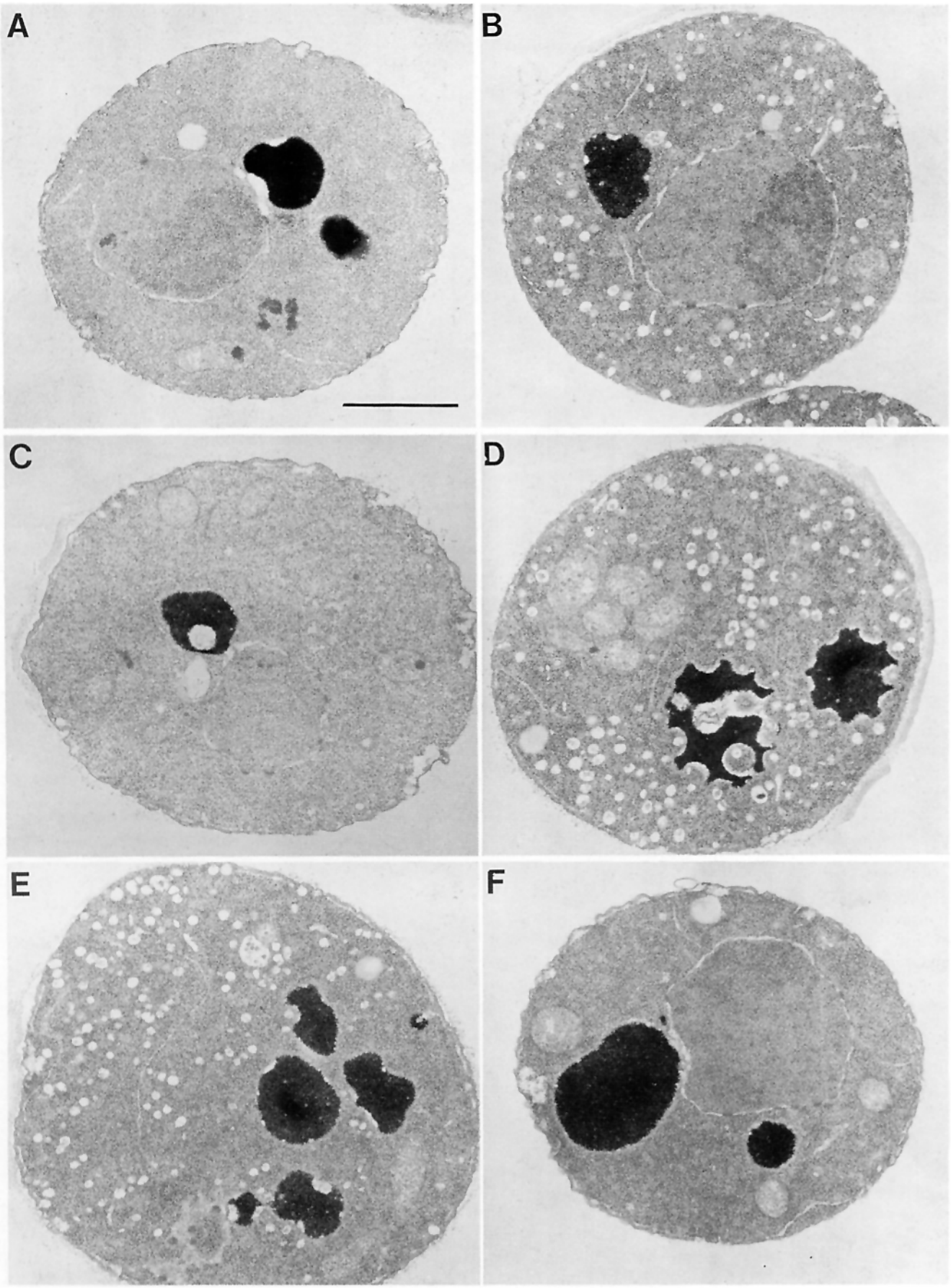
A
B
C
D
E
F

Table IV. Suppression of sec2 Secretion Defect by SEC4 Overexpression at 34°C

Strain	*sec*	Suppressor plasmid	Invertase Activity (μM glucose/min·A_{600}U) External 0 h	External 1 h	Internal 0 h	Internal 1 h	Invertase secretion (%)
NY 13	*SEC2*	–	.016	.402	.051	.055	99
NY 718	*SEC2*	pNB 139	.022	.271	.047	.051	98
NY 719	*SEC2*	pNB 170	.028	.307	.046	.054	97
NY 26	*sec2-59*	–	.030	.237	.039	.348	40
NY 720	*sec2-59*	pNB 139	.021	.369	.040	.237	64
NY 721	*sec2-59*	pNB 170	.021	.375	.043	.139	79
NY 130	*sec2-41*	–	.025	.329	.038	.317	52
NY 722	*sec2-41*	pNB 139	.024	.325	.041	.127	78
NY 723	*sec2-41*	pNB 170	.018	.356	.039	.113	82

sequence and 11 from read through into the adjacent pBR322-derived vector region, as determined from insert orientation and examination of *SEC2* and vector sequences. This protein, missing the last 251 amino acids of the *SEC2* gene product, migrates at 58 kD on immunoblots of NY673 lysates (Fig. 4), as expected from its sequence. More importantly, NY673 exhibits no temperature-sensitive defect, growing normally at 37°C. As expected from its good growth, *sec2-70* shows only slight decrease in secretion, down to 90% (Table III), confirming that removal of the last 251 amino acids does not create a temperature-sensitive allele of *SEC2*.

As removal of the last 251 amino acids did not create a conditional lethal defect, we constructed a second allele in which a greater portion of the protein was deleted. An 0.867-kb Sau IIIA–Sau IIIA fragment, extending from 306 to 1,173 bp, was inserted into YIp5. This plasmid, pNB298, was linearized at the internal Xba I site and used to transform a haploid strain (NY15) and a diploid strain (NY363). As before, transformants were selected on SD medium at 25°C. Homologous recombination produced a gene duplication in which the carboxy terminal deletion, *sec2-71*, lacks the last 368 amino acids of the Sec2 protein (Fig. 1 *C*, line 3). In this instance, the haploid transformants (NY688) exhibited severely restricted growth at 25°C, and at 37°C the allele proved lethal. On immunoblots of lysates from the diploid transformant (NY701), anti-Sec2 antisera recognized a 46-kD band (Fig. 4), as expected for the 429 amino acid protein encoded by the *sec2-71* gene: *SEC2* contributes 391 amino acids, and the final 38 residues originate from read through into the vector, as determined by analysis of the insert orientation and the insert and vector sequences. Because the haploid *sec2-71* strain, NY688, is nearly inviable at 25°C, immunoblot and secretion analysis was not performed.

Although *sec2-71* encodes a protein that is somewhat longer (391 amino acids of Sec2) than that encoded by *sec2-59* (374 amino acids), the *sec2-71* mutation causes a more severe growth defect at 25°C than does the *sec2-59* mutation. There are several possible explanations for this finding. Immunoblot analysis (Fig. 4) suggests that the *sec2-71* mutation not only truncates the protein but also lowers the level of expression. Thus, the combined effects of truncation and lowered expression may be more severe than the somewhat greater truncation resulting from the *sec2-59* mutation. Another possibility is that the 38 amino acids at the carboxy terminus that result from read through into vector sequence impede function of the *sec2-71* protein. Finally, there may be a low level of full-length Sec2 protein made in the *sec2-59* strain as a consequence of occasional read through of the opal codon. This low level of full length protein could enhance the growth rate at 25°C, but still be insufficient to sustain the growth at 37°C. No evidence for any full length protein can be seen by immunoblot analysis (Fig. 4); however, sensitivity of detection may be limiting.

Duplication of SEC4 Suppresses the Secretion Block in sec2-41 or sec2-59, but Does Not Alter the Expression of the Mutant Protein or Suppress a sec2 Null Mutation

In a previous report, we demonstrated that duplication of the *SEC4* gene could partially suppress the growth defect resulting from the *sec2-41* mutation (Salminen and Novick, 1987). We have investigated the nature of this phenomenon in greater detail. Two different plasmids were introduced into wild-type and *sec2* mutant strains: pNB139 yields approximately normal expression of *SEC4* thereby duplicating the total Sec4 protein level, while pNB170 yields severalfold higher than normal expression (Walworth et al., 1989). At 34°C both *sec2-41* and *sec2-59* exhibit a partial block in invertase secretion (Table IV). Introduction of either pNB139 or pNB170 allows increased efficiency of secretion. A somewhat greater effect is seen with pNB170 than with pNB139, as is consistent with the higher level of Sec4 protein expression. Thin-section electron microscopy demonstrated that pNB170 suppressed the vesicle accumulation phenotype of *sec2-41* at 34°C (Fig. 6, *E* and *F*). These results indicate that increased expression of *SEC4* suppresses the growth defect of *sec2* mutants by suppressing the secretory block rather than by allowing the cells to tolerate the secretory block.

Suppression of *sec2* mutations by increased expression of *SEC4* could be the result of increased expression or increased stability of the *sec2* mutant protein. To test this possibility, lysates were prepared from *sec2* mutants with and without plasmids carrying an extra copy of *SEC4*. Fig. 7 demonstrates that there is no detectable change in the level

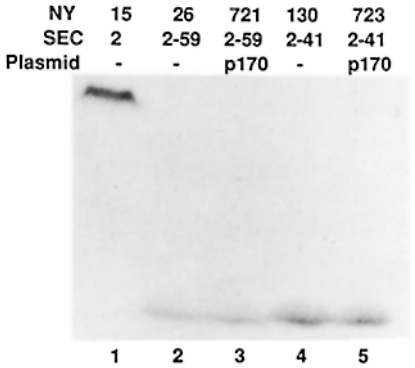

Figure 7. Anti-Sec2 immunoblot of *sec2-41* and *sec2-59* strains with and without an extra copy of *SEC4*. (Lane *1*) NY 15, a wild-type strain. (Lane 2) NY26, a *sec2-59* strain. (Lane *3*) NY721, a *sec2-59* strain carrying an episomal copy of *SEC4*. (Lane *4*) NY130, a *sec2-41* strain. (Lane *5*) NY723, a *sec2-41* strain carrying an episomal copy of *SEC4*.

Table V. Growth Properties of Single and Double sec Mutants

	sec				(*sec*, *sec2-41*)			
	25°C	30°C	33.5°C	37°C	25°C	30°C	33.5°C	37°C
sec1-1	+	+	−	−	+	−	−	−
sec2-41	+	+/−	−	−				
*sec3-2**	+	+	+	−/+	−	−	−	−
*sec4-8**	+	+	−/+	−	−	−	−	−
*sec5-24**	+	+	−/+	−	−	−	−	−
sec6-4	+	+	−	−	+/−	−	−	−
sec7-1	+	+	+	−	+	−/+	−	−
*sec8-9**	+	+	+	−	−	−	−	−
sec9-4	+	+/−	−	−	+/−	−	−	−
*sec10-2**	+	+	+	−	−	−	−	−
sec12-4	+	+/−	−	−	+	−	−	−
sec13-1	+	+/−	−	−	+	−	−	−
sec14-3	+	+	+	−	+	+/−	−	−
*sec15-1**	+	+	+	−/+	−	−	−	−
sec16-2	+	−/+	−	−	+	−/+	−	−
sec17-1	+	+	+/−	−	+	+/−	−	−
sec18-1	+	−	−	−	+	−	−	−
sec19-1	+	+	−	−	−/+	−	−	−
sec20-1	+	+/−	−	−	+	+/−	−	−
sec21-1	+	+	+	−	+	+/−	−	−
sec22-3	+	+/−	−	−	+	+/−	−	−
sec23-1	+	+/−	−	−	+	−/+	−	−

*Crosses in which the double mutants were inviable.

of the Sec2 mutant protein as a result of *SEC4* overexpression. This result indicates that stabilization or increased expression of Sec2 protein is not the basis of the observed suppression by Sec4 overproduction.

Suppression of *sec2* mutations by overexpression of *SEC4* could also be the result of a bypass of Sec2 protein function. This mechanism should allow suppression of a null allele. To test this possibility one of the *SEC2* genes in a *ura3-52/ura3-52, leu2-3,112/leu2-3,112* diploid strain was disrupted with the *URA3* gene (Fig. 1 *C, bottom line*). The resulting *sec2-72/SEC2* strain was subsequently transformed with a plasmid carrying the *LEU2* and *SEC4* genes (pNB318, Table II) by directing integration into the *SEC4* locus. This strain, carrying one duplicated copy of *SEC4* and one disrupted copy of *SEC2*, was subjected to tetrad analysis and spores were allowed to germinate at both 25°C and 14°C. If duplication of *SEC4* could suppress a *sec2* null allele then some tetrads should have contained three or four viable spores including one or two Ura$^+$, Leu$^+$ products. This was not seen. All tetrads contained only two viable spores, none of which were Ura$^+$, although many were Leu$^+$. This result establishes the fact that duplication of *SEC4* cannot suppress a null allele of *sec2*, ruling out the bypass mechanism of suppression, and furthermore implies that suppression of *sec2-41* requires the residual activity of the truncated protein at the elevated temperature.

sec-41 Is Lethal in Combination with a Subset of Late sec Mutants

In a previous study, we showed that *sec4-8* could not be combined in a haploid strain with mutations in any of a set of *sec* genes (Salminen and Novick, 1987). This set consisted of six of the nine other *sec* genes whose products are required for post-Golgi events, and it includes the *SEC2* gene. To extend this study we crossed a *sec2-41* strain to strains containing mutations in each of the late-acting *SEC* genes. The diploids were sporulated, tetrads were dissected, and allowed to germinate at 25°C. Lethality of about one quarter of the spores was seen in six crosses (Table V). The viable spores were tested for temperature-sensitivity. The pattern of inviability and temperature-sensitivity was consistent with the hypothesis that the double mutants are inviable in these cases. When all four spores were viable, they were all temperature-sensitive; when three were viable, two were temperature-sensitive, and when two were viable, they were both temperature-resistant. The lethal combinations are *sec2-41* with *sec3-2*, *sec4-8*, *sec5-25*, *sec8-9*, *sec10-2*, or *sec15-1*. The same set of mutations that are lethal when combined with *sec2-41* were previously found to be lethal when combined with either *sec4-8* and *sec15-1* (Salminen and Novick, 1987). The only exception is *sec19-1*. This mutation is inviable in combination with *sec4-8*, but viable in combination with *sec15-1* (Salminen and Novick, 1987). Combination of *sec19-1* with *sec2-41* results in viable colonies at 25°C; however, their growth is severely restricted.

Sec2 Protein Is Present in a Soluble, High Molecular Mass Complex

If, as predicted by its sequence, Sec2 protein interacts with itself or with another polypeptide, then it should display a native molecular mass significantly higher than that of the SDS-denatured protein. To test this possibility, a lysate was prepared by osmotic lysis of yeast spheroplasts and fractionated by the scheme described in Goud et al., 1988. The unbroken cells were cleared by centrifugation at 450 *g* for 5 min. This lysate was then spun at 10,000 *g* for 10 min, and the supernatant of that spin was spun at 100,000 *g* for 1 h. Immunoblot analysis of the supernatant and pellet fractions indicated that 6% of the total Sec2 protein (total is defined as that present in the 450 *g* supernatant) was pelletable at 10,000 *g*, 9% was pelletable at 100,000 *g*, and 50% was soluble (Fig. 8). This soluble fraction was applied to a Sephacryl S 300 HR (Pharmacia Fine Chemicals, Piscataway, NJ) column and eluted in 20 mM Hepes (pH 7.5), 50 mM NaCl,

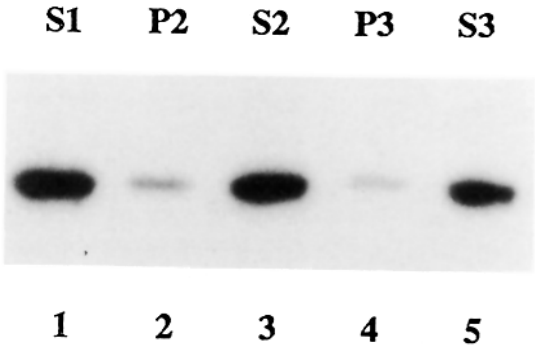

Figure 8. Solubility properties of Sec2 protein. Anti-Sec2 immunoblot analysis of samples derived from differential centrifugation of a wild-type (NY13) lysate. (Lane *1*) 450 *g* supernatant. (Lane *2*) 10,000 *g* pellet. (Lane *3*) 10,000 *g* supernatant. (Lane *4*) 100,000 *g* pellet. (Lane *5*) 100,000 *g* supernatant.

1 mM DTT. The distribution of Sec2 protein was assessed by immunoblot analysis and compared to the elution pattern of molecular mass standards (data not shown). A native molecular mass of ~500–750 kD was established. Analysis of a Sec2-41 lysate was attempted; however, the truncated protein was apparently unstable and no molecular mass determination could be made.

Discussion

We have presented an analysis of *SEC2* and its protein product. A major finding from these studies is that the Sec2 protein can be viewed as having two domains. The amino terminal half of the protein is essential and contains a peptide sequence that is predicted to be in an α-helical, coiled-coil conformation, while the carboxy terminal half of the protein is largely dispensable at 25°C. We have also probed the mechanism by which overproduction of Sec4 protein yields suppression of *sec2* mutants.

During the initial isolation of secretory mutants, mutations in the *sec2* gene were greatly overrepresented. Of 190 mutants that were defective in posttranslational events of the secretory pathway, 69 were found to be defective in the *sec2* gene, while the remaining mutants defined 22 additional complementation groups (Novick et al., 1980). The reason for this overrepresentation is now clear. Any nonsense mutation that truncates the Sec2 protein in the appropriate region will lead to a temperature-sensitive secretory defect. The critical region of Sec2 appears to lie between amino acids 374 and 391. This situation is in striking contrast to the *SEC4* gene, where three independently isolated mutations were all found to encode the same change of glycine at position 147 to aspartate, suggesting that the range of targets for this gene is much more restricted (Salminen and Novick, 1987; Walworth, unpublished data).

Our finding that the carboxy terminal half of Sec2 is dispensable has allowed us to focus more intensively on the amino terminal half of the protein for clues to the function of Sec2. Homology searches revealed significant similarity of this region of Sec2 to a number of cytoskeletal proteins including myosin and tropomyosin. Since previous studies have suggested a role for the actin based cytoskeleton in vesicular transport at the post-Golgi apparatus stage of the yeast secretory pathway (Novick and Botstein, 1985), one could postulate that Sec2 interacts with the cytoskeleton to facilitate vesicle transport. However, we believe that the sequence similarity of Sec2 with cytoskeletal proteins may simply reflect the fact that this region of Sec2, like many cytoskeletal proteins, exists as a coiled-coil α-helix, and it does not necessarily imply that Sec2 is itself cytoskeletal in nature. Supporting our hypothesis that Sec2 is complexed either with itself or with other components, we find that it has a very large apparent native molecular mass. Although Sec2 is predominantly soluble under the conditions tested, further studies will be needed to determine if the Sec2 complex is normally associated with a subcellular structure such as the cytoskeleton or an organelle.

The studies presented here allow a sharper definition of the interactions between *SEC2* and some of the other late acting *SEC* genes. Previous work has shown that duplication of the *SEC4* gene will suppress the growth defect of the temperature-sensitive *sec2-41* allele (Salminen and Novick, 1987). We now show that overexpression of *SEC4* will partially suppress all of the defects of *sec2-41*, and yet will not suppress a disruption of *SEC2*. Furthermore, overexpression of *SEC4* does not result in overexpression or stabilization of the mutant *sec2-41* gene product. These results are most consistent with a model in which the Sec4 protein acts as an upstream activator of Sec2 protein function. Sec4 was also invoked as an upstream activator of Sec15 protein function to explain similar data regarding the suppression of the *sec15-1* mutation by overexpression of *SEC4* (Salminen and Novick, 1989). A number of other genes may also be involved in this functional interaction. Mutations in *sec2*, *sec4*, and *sec15* exhibit synthetic lethality with each other, and in each case they also show synthetic lethality with mutations in *sec3*, *sec5*, *sec8*, and *sec10*.

The physical basis for the observed genetic interactions is not yet clear. Biochemical studies suggest that components must exist to regulate both the exchange of GTP onto Sec4 as well as to stimulate hydrolysis of the bound nucleotide (Kabcenell et al., 1990). Several of the interacting genes may encode such elements and biochemical analysis of the mutant strains may reveal these relationships. The enzymatic cycle of GTP binding and hydrolysis may be coupled to a topological cycle of Sec4 attachment to vesicles, exocytotic fusion, and recycling of Sec4 to new vesicles (Goud et al., 1988; Bourne, 1988; Walworth et al., 1989). By this model, vesicular Sec4 serves to direct the association of vesicles with the plasma membrane. This docking event may require the assembly of a complex of additional components. Hydrolysis of the bound GTP would allow disassembly of the complex for reutilization. The products of the interacting genes are candidates for components that act in response to Sec4 to control the interaction of vesicles with the plasma membrane. Overproduction of Sec4 may increase its concentration on the vesicle surface and by recruiting additional components to the docking complex help to overcome a partial defect in one of these downstream elements. Further studies on the interaction of these proteins with one another and with the compartments of the secretory pathway will allow us to test these proposals.

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