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Extragenic Suppressors of Mutations in the Cytoplasmic C Terminus of *SEC63* Define Five Genes in *Saccharomyces cerevisae*

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ABSTRACT

Mutations in the *SEC63* gene of *Saccharomyces cerevisiae* affect both nuclear protein localization and translocation of proteins into the endoplasmic reticulum. We now report the isolation of suppressors of *sec63-101* (formerly *npl1-1*), a temperature-sensitive allele of *SEC63*. Five complementation groups of extragenic mutations, *son1–son5* (suppressor of npl1-1), were identified among the recessive suppressors. The *son* mutations are specific to *SEC63*, are not bypass suppressors, and are not new alleles of previously identified secretory (*SEC61, SEC62, KAR2*) or nuclear protein localization genes (*NPL3, NPL4, NPL6*). *son1* mutations show regional specificity of suppression of *sec63* alleles. At low temperatures, *son1* mutants grow slowly and show partial mislocalization of nuclear antigens. The *SON1* gene maps to chromosome *IV* and encodes a nuclear protein of 531 amino acids that contains two acidic stretches and a putative nuclear localization sequence. We show that *son1* mutations suppress *sec63-101* by elimination of Son1p function.

CORRECT assembly of the nucleus is dependent upon accurate localization of nuclear proteins. Putative components of the nuclear protein localization machinery have been identified by a variety of biochemical methods: association with nuclear pores (GERACE, OTTAVARIO and KONDOR-KOCH 1982; DAVIS and BLOBEL 1986; HURT 1988; SNOW, SENIOR and GERACE 1987; ARIS and BLOBEL 1989; DAVIS and FINK 1990), nuclear localization sequence (NLS) binding capacity (ADAM *et al.* 1989; BENDITT *et al.* 1989; LEE and MELESE 1989; LI and THOMAS 1989; SILVER, SADLER and OSBORNE 1989; YAMASAKI, KANDA and LANFORD 1989; IMAMOTO-SONOBE *et al.* 1990; MEIER and BLOBEL 1990; ADAM and GERACE 1991; PANDEY and PARNAIK 1991), necessity in *in vitro* assays (ADAM, STERNE-MARR and GERACE 1990; NEWMEYER and FORBES 1990; ADAM and GERACE 1991; FINLAY *et al.* 1991; STOCHAJ *et al.* 1991; MOORE and BLOBEL 1992; STOCHAJ and SILVER 1992), and the ability to bind wheat germ agglutinin (FINLAY *et al.* 1987). Because the yeast *Saccharomyces cerevisiae* is particularly amenable to genetic analysis, SADLER *et al.* (1989) designed a screen to isolate temperature-sensitive (Ts^-) mutants defective in nuclear protein localization. Identification of the mutants was dependent on mislocalization of a nuclear-targeted fusion protein. Two *NPL* (*nu*clear *p*rotein *l*ocalization) genes identified in this screen have been cloned and further characterized: *NPL1* (= *SEC63*; see below) and *NPL6* (A. CHIANG and P. SILVER, unpublished results). A similar scheme, using a different reporter protein, resulted in the identification of *npl3* and *npl4* (BOSSIE *et al.* 1992; C. DEHORATIUS and P. SILVER, unpublished results).

npl1 is allelic to *sec63*. *sec63-1* was identified as a Ts^- mutant that fails to properly translocate secretory proteins into the endoplasmic reticulum (ER) (ROTHBLATT *et al.* 1989). Another Ts^- allele of *SEC63*, *ptl1* (*p*rotein *t*rans*l*ocation-defective), was independently isolated by TOYN *et al.* (1988) in a selection for ER translocation mutants. *SEC63* is an essential gene that encodes a protein of 663 amino acids (aa). Sec63p contains three potential membrane-spanning domains, an acidic C terminus (26 of the last 47 aa are either Asp or Glu), and a 70-aa region with similarity to the bacterial DnaJ protein (SADLER *et al.* 1989). In *Escherichia coli*, DnaJ interacts with an Hsp70 homolog, DnaK (LIBEREK, GEORGOPOULOS and ZYLICZ 1988; DODSON, MCMACKEN and ECHOLS 1989; ZYLICZ *et al.* 1989). Sec63p is localized to the nuclear envelope/ER network and behaves as an integral membrane protein (FELDHEIM, ROTHBLATT and SCHEKMAN 1992; KURIHARA and SILVER 1992). Topological analysis indicates that the C-terminal portion of Sec63p is cytoplasmic while the DnaJ domain resides in the ER lumen, where it may interact with Kar2p, the ER-localized Hsp70 homolog of yeast (FELDHEIM, ROTHBLATT and SCHEKMAN 1992; KURIHARA and SILVER 1992; NORMINGTON *et al.* 1989; ROSE, MISRA and VOGEL 1989).

The isolation of the *npl1*, *ptl1* and *sec63* alleles suggests that mutations in *SEC63* affect assembly of proteins into both the ER and the nucleus. Additional genetic and biochemical evidence (ROTHBLATT *et al.* 1989; DESHAIES *et al.* 1991) supports a role for Sec63p in ER protein translocation. It is not clear, however, that Sec63p participates directly in the process of

nuclear protein localization. Mutations in *SEC63* might alter the integrity of the nuclear envelope, thereby affecting the net accumulation of proteins in the nucleus.

In order to genetically identify additional components involved in protein trafficking, and to gain a better understanding of the relative role of Sec63p in ER translocation and nuclear protein localization, we have isolated suppressors of *sec63-101* (= *npl1-1*; see below). We now report the characterization of five mutations, *son1–son5* (suppressor of *npl1-1*). Molecular analysis of the *sec63* alleles allows us to correlate the suppression spectrum of *son1* and *son2* with topological domains of Sec63p. One gene, *SON1*, has been cloned and sequenced. *son1* mutants grow slowly at low temperatures and show a partial defect in nuclear protein localization.

MATERIALS AND METHODS

Strains and media: The yeast strains used in this study are listed in Table 1. Genetic manipulations were performed essentially as described in ROSE, WINSTON and HIETER (1990). See text and table legends for details of crosses. Because respiratory competence is necessary for sporulation [reviewed in ESPOSITO and KLAPHOLZ (1981)], a plasmid-borne copy of the *CYT1* gene was introduced into *cyt1::HIS3/cyt1::HIS3* diploids [either pIS41 (SADLER *et al.* 1989) or pAC1, a *URA3* marked derivative of pIS41].

Yeast media are described in ROSE, WINSTON and HIETER (1990). Auxotrophies and mating type were scored by spotting cell suspensions onto appropriate plates using a 48-prong inoculator (Dan-Kar Scientific, Reading, Massachusetts). Relative growth rates on different carbon sources or at different temperatures were determined by examining the size of colonies arising from streaks of single cells. Following its original detection at 14°, the slow-growth phenotype of the suppressor-bearing strains was found to be most easily scored by examining the colony size of cells grown for 2–3 days at 23°.

Unless otherwise noted, W303**a**Δ*cyt1* and W303αΔ*cyt1* were used as wild-type strains. After the initial isolation and characterization of the revertants, all crosses involving *sec63-101* were made to PSY76 or PSY327. In most cases, *SEC63 son* and *sec63-101 son* strains used in genetic analyses were the products of at least two crosses into the W303Δ*cyt1* background. The presence of the *son* mutation in *SEC63 son* strains was verified via a cross to *sec63-101* and subsequent tetrad analysis. Haploid *sec63-101 son* strains are able to grow at 36° but produce temperature-sensitive (Ts^-) diploids when crossed to *sec63-101*. PSY320 and PSY322 are Leu^+ spores from the cross of PSY316 to PSY327. The identity of *sec63-101 son1::LEU2* spores was verified in the same manner as that of *sec63-101 son* spores.

E. coli strain DH5α (GIBCO/BRL, Gaithersburg, Maryland) was used for manipulations involving bacteria. Media and culture techniques were as described in MILLER (1972) and SAMBROOK, FRITSCH and MANIATIS (1989).

Mapping and molecular analysis of *sec63* alleles: Four derivatives of *SEC63* were constructed by inserting a *Xho*I linker at one of the following four sites: *Stu*I (−39), *Acc*I (+744), *Pst*I (+1845) or *Acc*I (+2069). (Nucleotide numbering is based on a scale in which +1 corresponds to the A of the initial ATG. The *SEC63* coding sequence ends at +1992.) The resulting *Xho*I sites were used as endpoints for creation of three deletions, Δ1, Δ2 and Δ3 (see Figure 1). Fragments containing (−320 to 5′ *Xho*I site) and (3′ *Xho*I site to +2380) were subcloned in a pairwise manner into YIp5 (BOTSTEIN *et al.* 1979; STRUHL *et al.* 1979) such that when the 5′ and 3′ *Xho*I sites were ligated together, the intervening region of *SEC63* was deleted (Figure 1). Prior to transformation into the *sec63* mutants, the plasmids were linearized by *Xho*I digestion.

Synthetic primers were used to polymerase chain reaction (PCR) amplify (SAIKI *et al.* 1988) overlapping *SEC63* fragments from yeast genomic DNA prepared from wild-type and mutant cells. To minimize the risk of detecting PCR-generated mutations, the PCR products were sequenced directly (F. RUSSO, personal communication), following purification from low melting point agarose (SeaPlaque Agarose; FMC BioProducts, Rockland, Maine).

Revertant isolation: In order to facilitate subsequent diploid selection, the *sec63-101* parent strains, PSY5 and PSY6, were transformed with either a *LEU2* [pIS144 or pIS157 (Sadler *et al.* 1989)] or a *URA3* (pAC1, a derivative of pIS157) marked plasmid. These transformants, in turn, were mutagenized as follows. Cells were grown (under conditions selecting for plasmid maintenance) to a density of approximately 1×10^7 cells/ml, harvested by centrifugation, and resuspended in 0.1 M sodium phosphate, pH 7, at a density of 1×10^8 cells/ml. One-milliliter aliquots of cells were incubated with 2 μl of ethyl methanesulfonate (EMS) for 60 min at 30°. Mutagenesis was stopped by adding 50 ml of 5% sodium thiosulfate to each 1 ml of EMS/cell mixture. Cells were diluted in sterile water and plated on minimal medium (to select for plasmid maintenance) at 36° at densities of 5×10^7 cells/plate, 1×10^7 cells/plate, and 1×10^6 cells/plate. EMS treatment resulted in approximately 70% killing. Spontaneous revertants were isolated from cells incubated in sodium phosphate with no EMS. All other procedures were identical to those followed for mutagenized cells.

Three separate mutagenesis experiments (one with PSY6 and two with PSY5) were performed. A total of 1.2×10^8 mutagenized (1×10^8 unmutagenized) PSY6 cells and 2.0 $\times 10^8$ mutagenized (1.5×10^8 unmutagenized) PSY5 cells were examined. Heat-resistant (Ts^+) mutants were observed at an average frequency of 4.5×10^{-4} among mutagenized cells. Spontaneous Ts^+ mutants arose at a frequency of approximately 1×10^{-6}.

Ts^+ colonies (1800) were isolated and streaked to single colonies on minimal medium at 36°. The 1625 strains (including 114 spontaneous mutants) that showed continued ability to grow under these conditions were chosen for further study (see RESULTS). The *sec63-101* strains used for backcrosses were transformed with either a *URA3* or *LEU2* marked plasmid, in order to allow for selection of diploids on synthetic medium lacking leucine and uracil. (As described above, the revertants also carry a *LEU2* or *URA3* plasmid.)

Analysis of candidate *SON* genes: The following centromere-based (CEN) plasmids were used in the analysis of candidate *SON* genes: YCpNPL3-3 [*NPL3, URA3*; BOSSIE *et al.* (1992)], Lib7.1 [*NPL4, URA3*; C. DEHORATIUS and P. SILVER (unpublished results)], pAC4 [*NPL6, URA3*; A. CHIANG and P. SILVER (unpublished results)], PMR397 (*KAR2, URA3*; M. ROSE), pCS15 (*SEC61, LEU2*; R. DESHAIES), and pRD15 (*SEC62, LEU2*; R. DESHAIES).

Bypass suppressor analysis: *sec63-101 son* representatives were crossed to W303**a** to produce *sec63-101/SEC63 son/SON* diploids. pIS525 [*sec63::URA3* plasmid; SADLER *et al.* (1989)] was cut with *Ssp*I and *Sph*I and transformed into

TABLE 1

Yeast strains

Strain	Genotype	Origin
W303a	*MATa ura3-1 leu2-3,112 his3-11,15 trp1-1 ade2-1*	A. TZAGOLOFF
W303aΔcyt1	*MATa ura3-1 leu2-3,112 his3-11,15 trp1-1 ade2-1 cyt1::HIS3*	A. TZAGOLOFF
W303αΔcyt1	*MATα ura3-1 leu2-3,112 his3-11,15 trp1-1 ade2-1 cyt1::HIS3*	—[a]
PSY3	*MATa SEC63::URA3* in W303Δ*cyt* background	Integration into W303aΔ*cyt1*
PSY5	*MATa sec63-101* in W303Δ*cyt* background	Original *npl1-1* mutant
PSY6	*MATα sec63-101* in W303Δ*cyt* background	PSY5 × W303αΔ*cyt1*
PSY76	*MATa sec63-101* in W303Δ*cyt* background	PSY6 × W303aΔ*cyt1*
PSY327	*MATα sec63-101* in W303Δ*cyt* background	PSY6 × W303aΔ*cyt1*
PSY27	*MATa sec63-102* in W303Δ*cyt* background	Original *npl1-2* mutant
PSY28	*MATa sec63-104* in W303Δ*cyt* background	Original *npl1-4* mutant
PSY29	*MATa sec63-105* in W303Δ*cyt* background	Original *npl1-5* mutant
PSY30	*MATα sec63-106* in W303Δ*cyt* background	Original *npl1-6* mutant
PSY191	*MATa ura3-52 leu2-3,112 trp1Δ901 lys2-801 suc2Δ9 Δatp2::LEU2 sec63-107*	Original *npl1-7* mutant
PSY199[b]	*MATα ura3 leu2-3,112 trp1 cyt1::HIS3 sec63-107*	PSY191 × W303aΔcyt1
PSY192	*MATa ura1 his4 trp1 ade1 ade2 lys2 sec63-108*	Original *npl1-8* mutant
PSY202[b,c]	*MATα ura1 leu2-3,112 his4 ade2 cyt1::HIS3 sec63-108*	PSY192 × W303αΔ*cyt1*
RSY151	*MATα ura3-52 leu2-3,112 pep4-3 sec63-1*	R. SCHEKMAN
PSY97[a]	*MATa ura3 leu2-3,112 cyt1::HIS3 sec63-1*	RSY151 × PSY3
MS176	*MATα ura3-52 kar2-159*	M. ROSE
RDM15-5B	*MATα ura3-52 leu2-3,112 ade2 pep4-3 sec61-2*	R. SCHEKMAN
PSY139	*MATa ura3 leu2-3,112 trp1-1 ade2 sec61-2*	RDM15-5B × W303aΔ*cyt1*
RDM50-94C	*MATα ura3-52 leu2-3,112 his4 sec62-1*	R. SCHEKMAN
PSY140[b]	*MATa ura3 leu2-3,112 trp1-1 ade2-1 cyt1::HIS3 sec62-1*	RDM50-94C × W303aΔ*cyt1*
PSY247	*MATα ura3-52 leu2-3,112 his4-519 suc2-Δ9 gal2 Δatp2::LEU2 npl3-328*	—[d]
PSY109	*MATa ura3-52 leu2-3,112 trp1-Δ901 lys2-80 suc2-Δ9 Δatp2::LEU2 npl4-1*	—[d]
PSY231	*MATα npl6-1* in W303Δ*cyt* background	—[e]
PSY103	*MATα ura3-52 leu2-3,112 ade2-101*	—[f]
MS2368	*MATa/MATα ura3-52/ura3-52 leu2-3,112/leu2-3,112 his3-Δ200/his3-Δ200 +/trp1Δ ade2-101/+*	M. ROSE
PSY299	*MATa/MATα sec63-101/sec63::URA3 son1-1/SON1* in (W303a × W303αΔ*cyt1*)	This study
PSY301	*MATa/MATα sec63-101/sec63::URA3 son2-1/SON2* in (W303a × W303αΔ*cyt1*)	This study
PSY302	*MATa/MATα sec63-101/sec63::URA3 son5-1/SON5* in (W303a × W303αΔ*cyt1*)	This study
PSY303	*MATa/MATα sec63-101/sec63::URA3 son3-2/SON3* in (W303a × W303αΔ*cyt1*)	This study
PSY304	*MATa/MATα sec63-101/sec63::URA3 son4-1/SON4* in (W303a × W303αΔ*cyt1*)	This study
PSY316	*MATa son1::LEU2* in W303a background	This study
PSY318	*MATa/MATα son1::LEU2/+* in MS2368	This study
PSY320	*MATα son1::LEU2* in W303Δ*cyt* background	This study
PSY322	*MATα sec63-101 son1::LEU2* in W303Δ*cyt* background	This study
PSY323	*MATα son1::URA3* in W303Δ*cyt* background	This study

[a] Derived from W303aΔ*cyt1* by mating-type switching.
[b] The *cyt1::HIS3* disruption makes these cells His$^+$. The allele (*his3-11,15* or *HIS3*) at the *HIS3* locus has not been determined.
[c] PSY202 is Ura$^-$ Ade$^-$. The alleles at the *URA3* and *ADE1* loci have not been determined. A plasmid-borne copy of *URA3* cannot rescue the strain for growth on media lacking uracil, indicating that PSY202 carries at least the *ura1* mutation.
[d] BOSSIE *et al.* (1992).
[e] A. CHIANG and P. SILVER (unpublished results).
[f] H. BLUMBERG and P. SILVER (unpublished results).

these diploids in order to disrupt one copy of *SEC63*. Ura$^+$ transformants were tested for the ability to grow at 36° since it seemed likely that *sec63-101/sec63::URA3 son/SON* strains would be Ts$^-$. Southern analysis was used to confirm the integration of *URA3* at the *SEC63* locus. Tetrad data (see RESULTS) suggest that both the *sec63-101* and *sec63::URA3* alleles are present in PSY299 and PSY301–PSY304.

Cloning of *SON1*: *sec63-101 son1-1* cells (PSY265) were grown in 50 ml of YEPD at 30° to a density of 1.7 × 10^7 cells/ml, transformed with 1.5 μg of the A6 pool from a YCp50-based genomic library (ROSE *et al.* 1987), and selected at 30° on synthetic medium lacking uracil. Transformants were replica plated to selective medium at 30° and 36°. Isolates that gave rise to colonies at 30° but not at 36° were streaked to single cells on selective medium at 23°, 30° and 36°. Of 11,000 transformants, one showed the growth phenotype expected for *son1-1* rescue in a *sec63-101* background (Ts$^-$ at 36°; normal growth at 23°). The plasmid isolated from this transformant was named pMN35. pMN41, the plasmid used for genomic integration, carries the same insert as pMN40 (see below and Figure 4B) but in a YIp5 backbone.

Deletion derivatives were used to define the region of pMN35 required for *SON1* complementing activity (Figure 4B). Digestion of pMN35 with *Cla*I produces fragments of approximately 15.5 and 3.5 kb. The 3.5-kb fragment was subcloned into the *Cla*I site of YCp50 (ROSE *et al.* 1987) to produce pMN40. pMN44 was created by reclosing the 15.5-kb fragment with DNA ligase. Digestion of pMN44 with

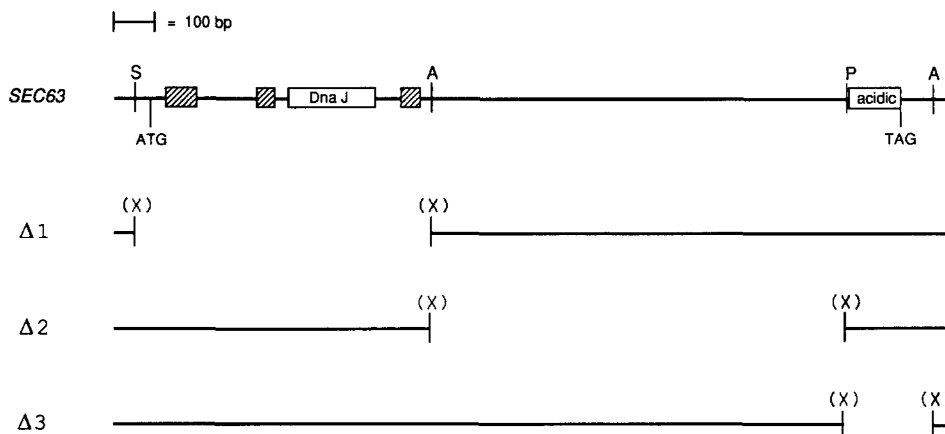


FIGURE 1.—Structure of *SEC63* deletion derivatives used in gap repair mapping analysis. The top line shows a partial restriction map of the *SEC63* region. Positions of the start (ATG; +1) and stop (TAG; +1992) codons are indicated below the line. Hatched boxes represent proposed membrane-spanning domains. The locations of the C-terminal acidic domain and of the region similar to DnaJ are also marked by boxes. S = *Stu*I (−39), A = *Acc*I (+744, +2069), P = *Pst*I (+1845). The lines in the maps of Δ1, Δ2 and Δ, 3 represent DNA present. (X) marks the site of *Xho*I linker insertion. See MATERIALS AND METHODS for details of plasmid construction.

*Bam*HI and subsequent reclosure produced pMN46. pMN43 was generated by *Pvu*II digestion of pMN35 followed by isolation and religation of the large backbone fragment.

Sequence analysis of *SON1*: A series of overlapping subclones inserted in Bluescript KS+ (Stratagene, La Jolla, California) was sequenced. DNA sequencing was performed using the dideoxy chain termination method of SANGER, NICKLEN and COULSON (1977), as modified for use with double-stranded template DNA (U.S. Biochemical, Cleveland, Ohio). Primers were either standard Bluescript primers or synthetic primers homologous to regions of *SON1*.

A minimal complementing fragment was PCR amplified (SAIKI *et al.* 1988) from pMN35. The primers for PCR were designed so as to create a *Sal*I site 389 bp 5′ of the predicted *SON1* open reading frame (ORF) and a *Hin*dIII site 266 bp 3′ of the ORF. The resulting 2.25-kb *Sal*I/*Hin*dIII fragment was cloned into YCp50 and YEp352 (HILL *et al.* 1986), producing pMN65 and pMN62, respectively.

***pMN71* construction:** SON1 was placed under control of the *GAL1* promoter (GUARENTE, YOCUM and GIFFORD 1982) by cutting pMN62 with *Kpn*I (which cuts in the YEp352 polylinker region 5′ of *SON1*) and *Xba*I (which cuts 19 bp upstream of the *SON1* ATG), isolating the vector backbone, and inserting the *Kpn*I/*Xba*I fragment from pKS-Gal (*GAL1* promoter in Bluescript KS+; D. ROOF and M. ROSE) to create pMN71.

Son1p-invertase fusions: A 2.0-kb *Sal*I/*Hin*dIII fragment, containing *SON1* and 386 bp of upstream sequence, was PCR amplified from pMN35. The 3′ PCR primer was designed to add a *Hin*dIII site in place of the *SON1* stop codon. This *Sal*I/*Hin*dIII fragment was cloned into YEp352 to generate pMN75. An in-frame fusion with *SUC2* was created by introducing the *Sal*I/*Hin*dIII fragment of pMN75 into pSEY304 that had been cut with *Hin*dIII and *Sal*I [pSEY304 is a 2μ-based *URA3* plasmid that contains a truncated form of *SUC2* missing its own signal sequence. A polylinker, which includes *Sal*I and *Hin*dIII sites, has been inserted at codon three of mature invertase (BANKAITIS, JOHNSON and EMR 1986)]. The resulting fusion protein should consist of 531 residues from Son1p, 3 residues at the junction and 511 residues from invertase. pMN94, a galactose-inducible *SON1-SUC2* fusion, was constructed as follows. pMN71 was cut with *Kas*I (cuts 150 bp 3′ of YEp352 polylinker region), blunted by treatment with the Klenow fragment of DNA polymerase I, and cut with *Xba*I to yield a YEp352-based backbone carrying the *GAL1* promoter. pMN94 was created by inserting a 1.6-kb *Xba*I/*Hin*dIII fragment from pMN75 (lacks 5′ *SON1* sequences and the *SON1* stop codon) and a *Hin*dIII/*Pvu*II *SUC2* fragment from pSEY304 into the pMN71 backbone. Son1p-invertase production was induced by the addition of galactose (to 2%) to cultures of pMN94 transformants that had been grown to midlog phase in selective medium plus 5% raffinose.

***SON1* null alleles:** The 2.25-kb *Sal*I/*Hin*dIII *SON1* region from pMN62 was cloned into pMN63, a modified version of YIp5 from which the *Eco*RI site has been eliminated, to produce pMN66. pMN67, a derivative of pMN66 lacking the *URA3* gene, was created by digestion with *Nde*I and subsequent reclosure. The *LEU2* gene was isolated from YEp351 (HILL *et al.* 1986) on a *Hpa*I/*Bam*HI fragment. This fragment was inserted into pMN67 DNA that had been cut with *Eco*RI, blunted by treatment with the Klenow fragment of DNA polymerase I, and cut with *Bam*HI. The resulting *son1::LEU2* construct was named pMN69. A 3.3-kb *Sma*I/*Dra*I fragment, which includes *LEU2* flanked by 500 bp of *SON1* at each end, was purified from pMN69 and transformed into W303**a** and MS2368. Stable Leu^+ transformants were analyzed via Southern blot to identify those with the predicted *son1::LEU2* disruption allele in their genome (data not shown).

pMN70 was constructed in the same way as pMN69 except that a *Hpa*I/*Bam*HI *URA3* fragment isolated from YEp352 was used in place of the *LEU2* marker. A 2.6-kb *Sma*I/*Dra*I fragment was purified from pMN70 and transformed into W303$\alpha\Delta$*cyt1*. Ura^+ transformants were analyzed via Southern blot to identify *son1::URA3* null alleles (data not shown).

Indirect immunofluorescence: Cells were prepared for indirect immunofluorescence as described in SADLER *et al.* (1989) with the following modifications. The amount of glusulase (Du Pont Co., Wilmington, Delaware) and Zymolyase (10 mg/ml Zymolyase 100,000; Miles Scientific Division, Naperville, Illinois) used to spheroplast the cells was reduced to 3 μl and 2 μl, respectively. Cells grown in the presence of galactose or raffinose were spheroplasted for 45 min (*vs.* 60 min for those grown in glucose). SV40-invertase was visualized using a 1:10,000 dilution of rabbit anti-invertase, followed by treatment with a fluorescein isothio-

TABLE 2

***sec63* alleles**

sec63 allele	Original name	Origin of mutant
sec63-1	*sec63-1*	ROTHBLATT *et al.* (1989)
sec63-101	*npl1-1*	SADLER *et al.* (1989)
sec63-102	*npl1-2*	SADLER *et al.* (1989)
sec63-104	*npl1-4*	SADLER *et al.* (1989)
sec63-105	*npl1-5*	SADLER *et al.* (1989)
sec63-106	*npl1-6*	SADLER *et al.* (1989)
sec63-107	*npl1-7*	BOSSIE *et al.* (1992)
sec63-108	*npl1-8*	KLYCE and MCLAUGHLIN (1973)
	ptl1	TOYN *et al.* (1988)

cyanate (FITC)-conjugated goat anti-rabbit IgG (Miles Scientific Division, Naperville, Illinois, or Jackson ImmunoResearch Laboratories, Inc., West Grove, Pennsylvania) at a 1:1000 dilution. To visualize histone H2B, a 1:700 dilution of rabbit anti-histone H2B (M. GRUNSTEIN) was used, followed by FITC-conjugated goat anti-rabbit as described above.

Other techniques: The lithium acetate method of ITO *et al.* (1983) was used for yeast transformations. DNA manipulations and Southern blot analyses were performed essentially as described in SAMBROOK, FRITSCH and MANIATIS (1989). Yeast genomic DNA was prepared by the method of ROSE, WINSTON and HIETER (1990). The PrimeClone blots were probed according to directions provided by L. RILES and M. OLSON. Probes for hybridization analyses were prepared from isolated DNA fragments using the BRL Random Primer kit. PCR amplification was performed in an Ericomp SingleBlock apparatus.

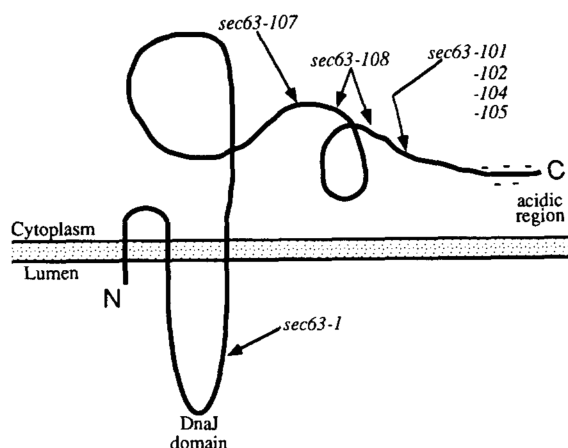
RESULTS

Mapping and molecular analysis of *sec63* alleles: Genetic analyses have identified at least nine temperature-sensitive (Ts$^-$) *sec63* alleles (Table 2). *sec63-1* was isolated as a *sec*retory mutant blocked at the point of protein translocation into the ER (ROTHBLATT *et al.* 1989). *npl1-1, npl1-2, npl1-4, npl1-5* and *npl1-6* were identified in an independent screen for mutants defective in *n*uclear *p*rotein *l*ocalization. The *npl* mutants were identified based on their ability to mislocalize a normally nuclear NLS-cytochrome c_1 fusion protein to the mitochondria (SADLER *et al.* 1989). A similar screen (BOSSIE *et al.* 1992) resulted in the isolation of *npl1-7*. *npl1-8* was isolated from a Ts$^-$ collection (KLYCE and MCLAUGHLIN 1973) as a mutant unable to complement the Ts$^-$ defect of *npl1-1* in diploid analysis. *npl1-8* is Ts$^-$ at both 30° and 36°. For the purpose of consistency in genetic nomenclature, we have assigned the *npl1* alleles *sec63* designations, beginning with *sec63-101*. Thus *npl1-1* becomes *sec63-101*, *npl1-2* becomes *sec63-102*, etc. (see Table 2).

We used the technique of gap repair (ORR-WEAVER and SZOSTAK 1983) to genetically map several *sec63* alleles. Three derivatives of *SEC63*, lacking nucleotides −39 to +744 (Δ1; Figure 1), +744 to +1845 (Δ2; Figure 1), or +1845 to +2069 (Δ3; Figure 1) were subcloned into YIp5 (BOTSTEIN *et al.* 1979; STRUHL *et al.* 1979). According to the membrane topology predictions of FELDHEIM, ROTHBLATT and SCHEKMAN (1992), the N-terminal deletion (Δ1) encompasses the DnaJ-homologous region as well as all three membrane-spanning domains. The extreme C-terminal deletion (Δ3) removes the acidic tail of Sec63p. (Figure 2A shows the predicted membrane topology of Sec63p.) Linear fragments were transformed into *sec63-101, sec63-102, sec63-104, sec63-105, sec63-106, sec63-107* and *sec63-108*. Heat-resistant (Ts$^+$) transformants could arise in the process of gap repair/integration only if the genomic mutation did *not* lie within the missing region of *SEC63*. Strains transformed with either (Δ1) or (Δ3) gave rise to Ts$^+$ colonies. In contrast, all colonies derived from (Δ2) transformants were Ts$^-$. Thus the *sec63-100* alleles all map in the C-terminal portion of *SEC63*, upstream of nucleotide 1845.

In order to determine the molecular nature of these

A.



B.

sec63 allele	Mutation
sec63-1	Ala179→Thr
sec63-101 *-102* *-104* *-105*	Gly511→Arg
sec63-106	n.d.[a]
sec63-107	Pro426→Leu
sec63-108	Ile431→Asn Pro503→Ala

FIGURE 2.—(A) Location of various *sec63* mutations with respect to proposed Sec63p topology. (The loops in the cytoplasmic domain are not intended to imply any knowledge of tertiary structure.) (B) Amino acid changes resulting from *sec63* mutations. [a]Precise nature of *sec63-106* has not been determined. As defined by mapping analysis the mutation lies in the cytoplasmic domain of Sec63p. It is not the same mutation as *sec63-101, sec63-107* or *sec63-108*.

TABLE 3

Distribution of suppressors among six classes

Class	No. members	Suppression of *sec63-101*	Slow-growth phenotype
I	1234	Dominant	None
II	21	Dominant	Recessive
III	3	Dominant	Dominant
IV	333	Recessive	None
V	31	Recessive	Recessive
VI	3	Recessive	Dominant

TABLE 4

Recessive suppressors define at least four complementation groups

Group	No. members
SON1	39
SON2	4
SON3	4[a]
SON4	12

[a] *son3-1* was subsequently found to be unlinked to the other *son3* alleles and has been renamed *son5-1* (see text).

mutations, PCR (SAIKI *et al.* 1988) was used to amplify overlapping fragments from the C-terminal domain of the corresponding genomic DNAs. PCR-amplified fragments were directly sequenced (F. RUSSO, personal communication), using PCR primers as sequencing primers, to reduce the risk of sequencing PCR-induced errors in individual subclones. The DNA sequence of the PCR products was compared with the wild-type *SEC63* sequence; Figure 2B lists the differences observed. *sec63-101*, *sec63-102*, *sec63-104* and *sec63-105* all have the same point mutation (Gly-511 to Arg). *sec63-107* has a single change at residue 426 (Pro to Leu). *sec63-108* carries two point mutations, at amino acids 431 (Ile to Asn) and 503 (Pro to Ala). The complete sequence of the C-terminal portion of *sec63-106* has not been determined. It has, however, wild-type sequence at residues 426, 431, 503 and 511 and so must represent a different mutation.

We defined the mutation in *sec63-1* by direct sequencing of overlapping PCR products that spanned the entire length of the gene. A single mutation, at aa 179 (Ala to Thr), was found (Figure 2B). Unlike those of the *sec63-100* alleles, the *sec63-1* mutation lies in the predicted lumenal domain of Sec63p (Figure 2A). Ala-179 is a highly conserved residue within the region of similarity to DnaJ.

Isolation and initial classification of suppressors: We isolated 1625 pseudorevertants of *sec63-101* by direct selection of Ts$^+$ colonies at 36°. The revertants were sorted into six classes (Table 3) based on three criteria. First, the mutants were backcrossed to the *sec63-101* strain of the opposite mating type. If the resulting diploid was Ts$^-$, the suppressor mutation was judged to be recessive. Revertants that gave rise to Ts$^+$ diploids were classified as dominant. On this basis, 367 mutations were identified as recessive and 1258 as dominant. Second, the revertants were assayed for their ability to grow at 14°. Fifty-eight strains showed a reduced rate of growth at 14° in comparison to the *sec63-101* parent. Third, the mutation giving rise to the slow-growth phenotype was classified as dominant or recessive based on the ability of heterozygous diploids to grow at 14°. All but six mutations causing the slow-growth phenotype were recessive.

Initial analysis of recessive suppressors: Our objective in carrying out pseudoreversion analysis was to identify additional components of the cellular protein localization machinery. Thus, we were most interested in extragenic suppressors of *sec63-101*. Of 10 dominant suppressors subjected to additional genetic analysis, all appeared to be intragenic (based on the inability to recover any Ts$^-$ spores from diploids produced by crosses between the revertants and wild-type cells; data not shown). Therefore, only recessive suppressors were further characterized.

Complementation tests were carried out on a subset of revertants (from classes IV and V; see Table 3). Ts$^+$ isolates of opposite mating type were mated and the resulting diploids were tested for their ability to grow at 36°. If two strains produced a Ts$^-$ diploid, the two mutations were judged to complement one another. If a Ts$^+$ diploid was produced, the two strains were placed in the same complementation group. Of the 138 isolates tested in this manner, 59 fell into one of four complementation groups (Table 4). Since a complementation group whose members are all of the same mating type would not have been detected in this analysis, it is possible that one or more additional multiallelic complementation groups exist among the remaining 79 strains. The four complementation groups defined by these crosses have been designated *son1–son4*, for suppressor of npl1-1. (Recall that *sec63-101* was originally named *npl1-1*.)

Since unlinked noncomplementation is possible, we wished to determine whether the alleles of a given complementation group actually define a single locus. Representative *sec63-101 son* strains were crossed in pairwise combinations within each complementation group. If the two *son* mutations are truly allelic, all spores produced from such a cross should be Ts$^+$; if not, 25% of the resulting spores will be Ts$^-$. With the exception of *son3–1*, the linkage data confirmed the original complementation analysis. While *son3-2*, *son3-3* and *son3-4* are all linked to one another, *son3-1* is not linked to any of the other *son3* alleles (data not shown). Accordingly, *son3-1* has been renamed *son5-1*. *son3* and *son5* thus show unlinked noncomplementation.

In order to determine whether *son1–son5* are extra-

genic suppressor mutations, representative *sec63-101 son* strains were crossed to wild-type strains. In the case of an unlinked extragenic suppressor, 25% of the spores recovered from such a cross should be Ts^-. Furthermore, the resulting tetrads should show three different segregation patterns in a ratio of 1 parental ditype (PD):4 tetratype (T):1 nonparental ditype (NPD). Based on these criteria, all *son* representatives examined behave as single suppressor mutations, unlinked to the *SEC63* locus (data not shown).

Strains bearing *son1-1*, *son1-3* and *son2-2* were initially classified as slow-growing revertants. In crosses with wild-type cells (described above), the slow-growth phenotypes of *son1-1*, *son1-3* and *son2-2* segregated as single mutations. Moreover, all slow-growing spores were scored as Ts^+ at 36° consistent with linkage between the suppression and slow-growth phenotypes.

Analysis of candidate *SON* genes: Mutations in *SEC63* affect both nuclear protein localization (SADLER *et al.* 1989) and protein translocation into the ER (TOYN *et al.* 1988; ROTHBLATT *et al.* 1989). Thus, if the *son* mutants are new alleles of previously identified genes, the most likely candidates are either other *NPL* genes or other early *SEC* genes. This possibility was tested via plasmid complementation. Six candidate *SON* genes were tested in this manner: *NPL3* (BOSSIE *et al.* 1992), *NPL4* (C. DEHORATIUS and P. SILVER, unpublished results), *NPL6* (A. CHIANG and P. SILVER, unpublished results), *SEC61* (DESHAIES and SCHEKMAN 1987), *SEC62* (DESHAIES and SCHEKMAN 1989; ROTHBLATT *et al.* 1989), and *KAR2* (NORMINGTON *et al.* 1989; ROSE, MISRA and VOGEL 1989). Representative alleles of *son1–son5* were analyzed and all transformants were found to be Ts^+. Thus, the *son* mutations identify five novel genes, *SON1–SON5*.

Gene specificity: *son* mutations were tested for their ability to suppress mutations in other related genes. *SEC63 son* strains were crossed to *kar2-159* (VOGEL, MISRA and ROSE 1990) and the resulting diploid sporulated. Since none of the *son* mutations are alleles of *kar2*, the continued 2:2 segregation of *kar2*'s Ts^- phenotype indicated that none of the *son* mutations (*son1–son4*) suppress *kar2-159*. In analogous crosses, the Ts^- phenotypes of *sec61-2* (DESHAIES and SCHEKMAN 1987) and *sec62-1* (ROTHBLATT *et al.* 1989) also segregated 2:2. Hence, the ability of *son1–son4* to suppress *sec63-101* does not extend to other early secretory mutations. Similarly, further analysis indicated that *son1-1* displays specificity with regard to *npl* mutations. *son1-1* does not suppress *npl3-328* (BOSSIE *et al.* 1992), *npl4-1* (C. DEHORATIUS and P. SILVER, unpublished results), or *npl6-1* (A. CHIANG and P. SILVER, unpublished results). Thus, the *son* mutations are not omnipotent suppressors of protein mislocalization. Instead, they appear specific for alleles of *SEC63*.

TABLE 5

Allele specificity of *son* suppressors

son allele	Phenotype of double mutants (no. of tetrads scored)				
	sec63-101	*sec63-106*	*sec63-107*	*sec63-108*	*sec63-1*[a]
SON	Ts^-	Ts^-	Ts^-	Ts^-	Ts^-
son1-1	Ts^+	Ts^+ (32)	Ts^+ (23)	Ts^+ (20)[b]	Ts^- (15)
son2-1	Ts^+	—	—	—	Ts^- (12)
son3-2	Ts^+	—	—	—	Ts^+ (12)
son4-1	Ts^+	—	—	—	Ts^+ (12)

Tetrad data for *sec63-106*, *sec63-107*, *sec63-108* and *sec63-1* were derived from crosses of PSY30 (*sec63-106*), PSY199 (*sec63-107*), PSY202 (*sec63-108*) and PSY97 (*sec63-1*) to the appropriate *SEC63 son* strains. The phenotype of the double mutants was inferred by determining whether the resulting spores included tetratype (3 Ts^+:1 Ts^-) and nonparental ditype (4 Ts^+) tetrads (see text). See text for a description of the experiments involving *sec63::URA3*, which were all inviable.

[a] Because the original *sec63-1* strain, RSY151, has a second Ts^- mutation (38°) closely linked to *sec63-1*, a derivative strain, PSY97 was constructed. The only Ts^- mutation in PSY97 is at the *SEC63* locus (data not shown).

[b] *sec63-108* strains are Ts^- at 30° and 36°. *sec63-108 son1-1* mutants can grow at 30° but not at 36°.

Allele specificity: The *son* mutations are not bypass suppressors. We tested this possibility by sporulating *sec63-101/sec63::URA3 son/SON* diploids. *SEC63* is an essential gene. Therefore, if a *son* mutation is capable of suppressing *sec63::URA*, tetrads with two, three, and four viable spores should be observed (in a ratio of 1:4:1). In all five cases (*son1–son5*), however, only two viable spores could be obtained from any single tetrad (Table 5; data for *son5-1* not shown). All viable spores were Ura^-. Fifty percent of the spore clones were Ts^-, due to independent segregation of the *SEC63* and *SON* loci. Thus, although the suppressors are able to suppress *sec63-101* in this background, they cannot suppress a *sec63* null allele.

We further investigated the allele specificity of the *son* mutations. *SEC63 son* mutants were crossed with *sec63* strains. Segregants derived from the resulting *sec63/SEC63 SON/son* diploids were scored for growth at 36°. The data are summarized in Table 5. In the case of *sec63-1*, only 25% of the spores derived from crosses with *son3-2* and *son4-1* were Ts^-, indicating that these two *son* mutations can suppress *sec63-1*. Tetrads from similar crosses of *son1-1* and *son2-1* with *sec63-1*, on the other hand, show 2:2 segregation of the Ts^- phenotype. Thus *son1-1* and *son2-1* are able to suppress *sec63-101* but not *sec63-1*. In similar analyses, *son1-1* was found to suppress *sec63-106* and *sec63-107*. Its suppression of *sec63-108* is partial. *sec63-108 son1-1* strains grow at 30°, but are still Ts^- at 36°. Sequence and mapping data (see above) indicate that the *sec63-106*, *sec63-107* and *sec63-108* mutations all lie in a region of the locus different from that of *sec63-1*. Thus, the allele specificity of suppression by *son1-1* seems to be a regional specificity.

TABLE 6

Ability of NLS-*CYT1* transformants to grow on glycerol

son allele	*SEC63* allele	
	sec63-101	*SEC63*
SON	+	–
son1-1	+	–
1-2	+	(+)
1-3	+	–
son1::URA3	ND[a]	–
son2-1	+	–
2-2	+	–
son3-2	+	(+)
son4-1	–	–
4-2	–	–
son5-1	–	–

cyt1::HIS3 strains of the indicated genotypes were transformed with pIS157 [SV40 NLS-*CYT1*; Sadler *et al.* (1989)] and scored for their ability to grow at 30° with glycerol as their sole carbon source (YEP + 3% glycerol). "+" indicates colonies arising in 3 days. "(+)" indicated growth in 5 days.

[a] ND = not determined.

Growth on glycerol: *sec63-101* was originally isolated by SADLER *et al.* (1989) as a Ts^- mutant that, at the semipermissive temperature of 30°, mislocalized a normally nuclear NLS-cytochrome c_1 fusion protein to the mitochondria. (Cytochrome c_1 is a component of the mitochondrial respiratory chain.) In a *cyt1* background, mislocalization was detected by renewed ability of the mutants to grow on the nonfermentable carbon source glycerol (Gly^+). Gly^+, Ts^- cells were screened for mutants that show mislocalization of nuclear proteins when assayed by indirect immunofluorescence (SADLER *et al.* 1989). Since the Gly^+ and Ts^- phenotypes of *sec63-101* show linkage, we wondered whether any of the *son* mutations would affect the ability of *sec63-101* cells to grow on glycerol. As shown in Table 6, only *son5-1* and the *son4* alleles suppress *sec63-101*'s Gly^+ phenotype. Thus, in the cases of *son1*, *son2* and *son3*, it is possible to suppress the Ts^- phenotype of *sec63-101* without entirely correcting the protein sorting defect.

If the *son* mutations actually identify other components of the pathway(s) that *sec63-101* disrupts, then *son* mutations, in a *SEC63 cyt1* background, may cause phenotypes similar to those of the *sec63-101* mutant. Consequently, we assayed the ability of *SEC63 son* strains to grow on glycerol (Table 6). *son1-2* and *son3-2* are Gly^+, although their growth is weaker than that of *sec63-101 SON* strains. Curiously, *SEC63 son1-1*, *SEC63 son1-3* and *son1::URA3* (see below for characterization of *son1* null mutations) do not have a Gly^+ phenotype.

Nuclear protein localization in *son1* strains: Nuclear antigens are mislocalized in *SEC63 son1* mutants. In wild-type cells, a chimeric protein encoded by a fusion between the NLS from SV40 large T antigen and invertase (SV40-invertase) is efficiently targeted to the nucleus [NELSON and SILVER (1989) and Figure 3A]. In contrast, an increase in the level of cytoplasmic localization of SV40-invertase is observed in *SEC63 son1* mutants grown at 23° (Figure 3D). Moreover, 10–20% of *SEC63 son1* cells are unusually large and show aberrant nuclear morphology. [Figure 3 depicts the data for *son1* null mutants. (See below for characterization of *son1* null allele.) Similar results were obtained in experiments using *son1–1*; data not shown.] The SV40-invertase staining pattern, nuclear morphology, and cell size of *SEC63 son1* mutants grown at 36° is similar to that of wild-type cells; at 30°, an intermediate phenotype is observed (data not shown). Mislocalization is not specific to the SV40-invertase fusion protein since similar results are seen in cells stained with anti-histone H2B antibody (data not shown).

When grown at 30°, *sec63-101 SON1* and *sec63-101 son1* cells show a comparable degree of protein mislocalization (data not shown). As judged by indirect immunofluorescence therefore, *son1* does not appear to suppress the protein mislocalization phenotype of *sec63-101*. In fact, SV40-invertase localization is severely compromised in *sec63-101 son1* cells grown at 36° (Figure 3G). These results are consistent with our earlier observation that *sec63-101* mutants remain Gly^+ in a *son1-1* background (Table 6).

Cloning of *SON1*: *SON1* was cloned from a yeast genomic library (ROSE *et al.* 1987) by complementation. We reasoned that introduction of a plasmid encoding *SON1* into a *sec63-101 son1-1* strain would rescue both recessive growth phenotypes of *son1-1*. Thus, we expected *SON1* transformants to grow normally at 23° but, like *sec63-101*, to be Ts^- at 36°. Of approximately 11,000 *sec63-101 son1-1* transformants screened, one was found to have the predicted growth properties. Upon plasmid loss, the slow-growth and Ts^+ phenotypes of *sec63-101 son1-1* were restored. Plasmid DNA was isolated from the original yeast transformant and designated pMN35. When introduced into fresh *sec63-101 son1-1, sec63-101 son1-2,* and *sec63-101 son1-3* cells, this plasmid rescued both the slow-growth and Ts^+ phenotypes of the *son1* mutants. pMN35 was also capable of complementing the nuclear protein localization defect of *son1* mutants (data not shown).

Preliminary restriction mapping revealed that pMN35 carries an insert of approximately 11 kb (Figure 4A). Genomic integration was used to verify that pMN35 carries DNA from the *SON1* locus. pMN41 (a YIp5-based plasmid that carries the same insert as pMN40–see Figure 4B) was linearized by digestion with *Xba*I and transformed into *sec63-101*. Two stable Ura^+ integrants (verified by Southern analysis; data not shown) were crossed to *sec63-101 son1-1*. The resulting diploids were subjected to tetrad analysis. In

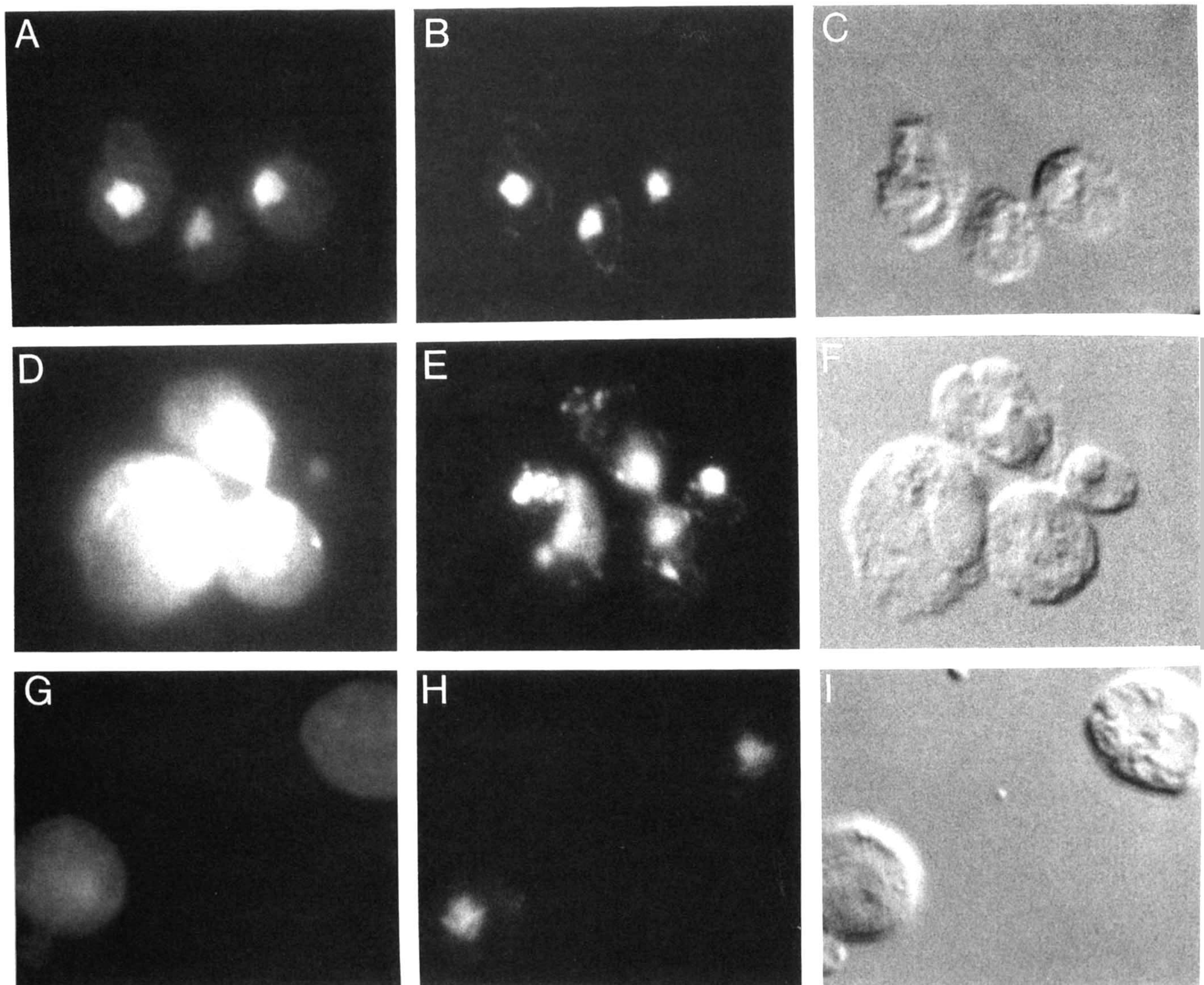


FIGURE 3.—Immunofluorescence localization of SV40-invertase. pMN8 (*SV40-SUC2* fusion from NELSON and SILVER 1989) transformants were grown at 23° or 36°, fixed, and processed for immunofluorescence. (A–C) W303αΔ*cyt1* grown at 23°. (D–F) PSY320 (*SEC63 son1::LEU2*) grown at 23". (G–I) PSY322 (*sec63-101 son1::LEU2*) grown at 36°. Panels A. D and G show cells treated with anti-invertase antibody and FITC-conjugated goat anti-rabbit IgG. 4,6-Diamidino-2-phenylindole (DAPI) staining was used to visualize DNA (B, E and H). Cells viewed by Nomarski optics are shown in panels C, F and I. Exposure times and degree of enlargement were identical for all strains.

all 43 tetrads examined, two spores were Ts^- and two were Ts^+. As expected for 2:2 segregation of *son1-1*, the Ts^+ spores also showed the slow-growth phenotype. In most cases the Ts^- spores were Ura^+, consistent with the hypothesis that integration had occurred at the *SON1* locus. Four tetratype tetrads were scored, however, indicating that *SON1* and the integrated copy of *URA3* are separated by about 4.6 cM. In fact, further analysis of pMN35 (see below and Figure 4B) revealed that pMN41 should direct integration of *URA3* approximately 1 1.4 kb from *SONl.*

The same 3.5-kb *Cla*I fragment contained in pMN41 was used to probe Primeclone Blots (OLSON *et al.* 1986). Hybridization to a single clone, #6718, was detected (data not shown). Comparison with the physical map generated from PrimeClone data (OLSON *et al.* 1986; L. RILES and M. OLSON, personal communication) indicates that clone #67 18 lies on the left arm of chromosome IV, between the clone carrying *CEN4* and the one carrying *RNA11* and *SIR2.* Consistent with these results, the *Cla*I fragment also hybridizes to chromosome *IV* (data not shown).

DNA sequence of *SON1*: Several deletion derivatives of pMN35 were generated and transformed into *sec63-101 son1-1* in order to assess their ability to complement the *son1-1* mutation. The complementation data (Figure 4B) defined a 0.9-kb *Pvu*II/*Bam*HI fragment critical for *SON1* complementing activity which, along with flanking regions, was sequenced. A single large ORF, encompassing the *Pvu*II/*Bam*HI fragment, was detected (Figures 4A and 5).

A fragment encoding the proposed ORF, as well as

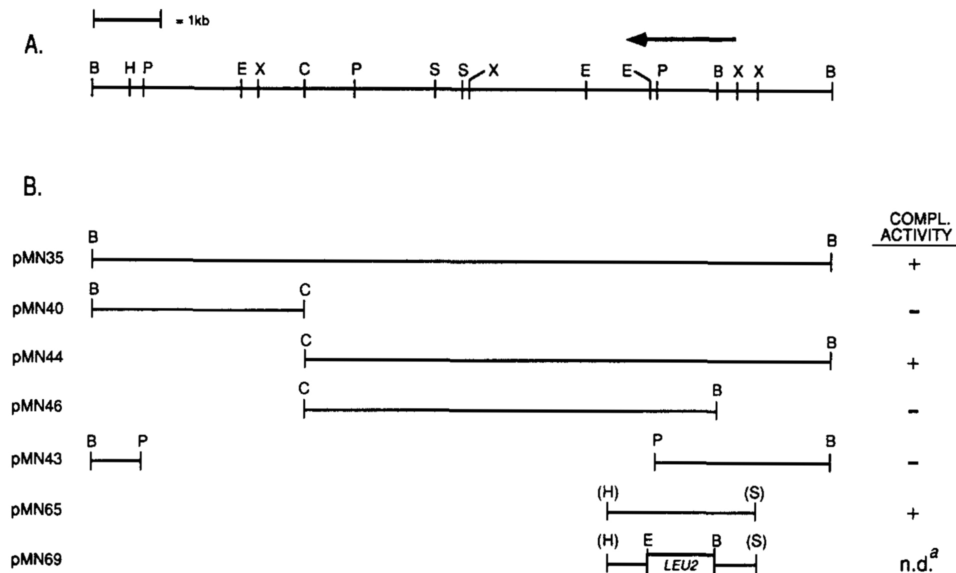


FIGURE 4.—(A) Partial restriction map of *SON1* region. Extent and direction of *SON1* ORF are shown by arrow above restriction map. B, *Bam*HI; C, *Cla*I; E, *Eco*RI; H, *Hin*dIII; P, *Pvu*II; S, *Sal*I; X, *Xba*I. (B) *SON1* complementation analysis. Lines show DNA present. Complementing activity was scored based on the ability to revert the Ts+ and slow-growth phenotype of *sec63-101 son1-1* (see text). pMN35 is the original library clone. pMN41, pMN44, pMN46, and pMN43 are various subclones of pMN35 (see MATERIALS AND METHODS). pMN65 carries the minimal *SON1* complementing fragment. (S) and (H) mark the *Sal*I and *Hin*dIII sites engineered onto the ends of the fragment during PCR amplification. pMN69 was the source of DNA used to create the *son1::LEU2* null allele. (*LEU2* not to scale.) [a]n.d. = not determined.

386 bp upstream and 266 bp downstream, was amplified (SAIKI *et al.* 1988) from pMN35 by PCR and cloned into YCp50 (ROSE *et al.* 1987) to create pMN65 (Figure 4B). When *sec63-101 son1-1* was transformed with pMN65, the plasmid complemented both the slow-growth and Ts+ phenotypes of *son1-1*. Since there is no other ORF in the amplified fragment, we conclude that this ORF is, in fact, the *SON1* coding sequence.

The *SON1* ORF predicts a protein of 531 aa (Figure 5) with a calculated molecular mass of 60 kD. Son1p contains two highly acidic stretches: aa 211–229 (14 of 19 residues) and aa 300–312 (11 of 13 residues). Amino acids 382–398 fit the consensus suggested by DINGWALL and LASKEY (1991) and ROBBINS *et al.* (1991) for a bipartite NLS: two basic residues, a spacer region of any 10 aa, and a group of five residues, of which at least three are basic. FastA analysis, using the UW-GCG program (DEVEREUX, HAEBERLI and SMITHIES 1984) revealed no significant homologies to the primary sequence of proteins in the database.

Subcellular localization of Son1p: In order to determine the subcellular localization of Son1p, we constructed a gene fusion between *SON1* and *SUC2*, the yeast gene for invertase (CARLSON and BOTSTEIN 1982; TAUSSIG and CARLSON 1983). The resulting fusion protein shows partial ability to complement *son1* but has no effect on the growth rate of wild-type cells. We were unable, however, to visualize the fusion protein with anti-invertase antibody via either indirect immunofluorescence or immunoblotting. Accordingly, we constructed a galactose-inducible *SON1-SUC2* fusion, pMN94. After 3 hr of growth in galactose, a protein of the predicted size (120 kD) was detected in cell extracts via immunoblotting with anti-invertase antibody. Maximal induction was observed by 6 hr (data not shown).

Following 6 hr of galactose induction and preparation for immunofluorescence using anti-invertase antibody, pMN94 transformants show predominantly nuclear staining (Figure 6, D and E). In contrast, only a low level of cytoplasmic staining is visible in pMN94 transformants grown in raffinose or in galactose-induced control cells (Figure 6, A and G). Since we know that, in the absence of a functional NLS, invertase is not nuclear-localized (NELSON and SILVER 1989), the accumulation of at least a portion of Son1p-invertase in the nucleus argues that Son1p itself is partially or completely localized to the nucleus, consistent with our observation of a putative NLS in the coding sequence (see above).

Creation of *son1* null alleles: In plasmid pMN69, *LEU2* has been placed between the *Bam*HI and *Eco*RI sites of *SON1*, thereby eliminating 323 aa from the center of Son1p (Figure 4B). Both haploid (W303**a**) and diploid (MS2368) yeast strains were transformed with a *SON1/LEU2* fragment from pMN69. Southern analysis (data not shown) confirmed that the haploid (PSY316) and the diploid (PSY318) carry the predicted *son1::LEU2* disruption. In tetrads derived from PSY318, all four spores were viable. As expected, two spores were Leu+ and two were Leu−. Since a viable

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SON1   CCTTTCCACGGATAATCACGTGATCGCTATTACTAATAGCCGTTTACAGTGATGGTTCTA   -344
                                                                  x
SON1   GAAAGTTCCGTTTCGGCGAAACTTGTTGAGGAGAGCAATTCTATATAAGGAAAGAATAGT   -284

SON1   TCGAAGCCCAATTTGGGGAAAGCCCGGGATGAGCAGCCTAACCCACACGGATAATTTTTT   -224
                               s
SON1   ATCGTTCAAGGAGGAGGATACGTTTTATCTCTAATATAAATTAAAGATAAACCCGAAACT   -164

SON1   CTTTTTTCTTCTTGGCGTGGAATATTTAAGGAGTAGATCAGTTCCATTTTGTTCATCCTT   -104

SON1   TTCTCCTCAAGGAAGAACTCCGCTTTTTAGTTGAACTAATAGTAGATAGTACTAATAGCT    -44

Son1p                                               M  A  S  T  E  L      6
SON1   AATTTGTATCTTTTCAAAAGTTTTCTAGAATTTTCAAGCAATCATGGCTTCTACGGAACT     17
                                x
Son1p  S  L  K  R  T  L  T  D  I  L  E  D  E  L  Y  H  T  N  P  G     26
SON1   TAGCCTAAAAAGAACCTTAACGGATATTTTAGAAGACGAGTTGTACCATACTAATCCAGG     77

Son1p  H  S  Q  F  T  S  H  Y  Q  N  Y  H  P  N  A  S  I  T  P  Y     46
SON1   TCACAGTCAGTTTACGAGTCATTATCAAAACTATCATCCAAATGCTAGTATTACTCCATA    137

Son1p  K  L  V  N  K  N  K  E  N  N  T  F  T  W  N  H  S  L  Q  H     66
SON1   TAAGTTGGTGAATAAGAACAAGGAAAACAACACTTTTACGTGGAATCATTCATTACAACA    197

Son1p  Q  N  E  S  S  A  A  S  I  P  P  Q  Q  T  Y  H  F  P  I  F     86
SON1   CCAGAATGAATCGAGTGCAGCTTCGATACCCCCACAACAAACCTACCATTTCCCGATATT    257

Son1p  N  K  Y  A  D  P  T  L  T  T  T  T  S  F  T  T  S  E  A  T    106
SON1   CAACAAATACGCGGATCCTACTTTAACTACCACCACCTCTTTTACGACTAGTGAAGCAAC    317
                  b
Son1p  A  N  D  R  Q  I  N  N  V  H  L  I  P  N  E  I  K  G  A  S    126
SON1   GGCCAACGATAGACAGATTAATAATGTCCATCTCATACCAAACGAGATTAAGGGTGCTAG    377

Son1p  E  T  P  L  Q  K  T  V  N  L  K  N  I  M  K  V  S  D  P  Y    146
SON1   CGAAACCCCATTGCAGAAGACCGTCAATCTAAAGAATATAATGAAAGTATCAGACCCGTA    437

Son1p  V  P  T  R  N  T  F  N  Y  D  V  K  I  S  N  D  F  F  D  N    166
SON1   TGTACCGACACGGAATACGTTCAATTATGATGTTAAAATTTCCAACGATTTTTTCGATAA    497

Son1p  G  D  N  L  Y  G  N  D  E  E  V  L  F  Y  E  D  N  Y  N  P    186
SON1   CGGTGACAATCTATATGGTAATGATGAAGAAGTGCTTTTCTATGAGGATAATTATAATCC    557

Son1p  K  M  Q  W  S  L  Q  D  N  S  A  A  I  N  N  E  D  A  R  A    206
SON1   GAAAATGCAGTGGTCACTTCAAGATAATAGCGCCGCAATAAACAATGAGGATGCGAGAGC    617

Son1p  I  F  N  N  E  F  D  S  D  D  D  D  I  S  D  D  E  E  D  E    226
SON1   TATTTTTAACAATGAATTTGACTCTGATGACGACGATATCAGTGATGATGAAGAGGATGA    677
                                           r
Son1p  I  E  E  N  C  L  Q  Q  E  Q  H  Q  E  E  P  L  L  S  L  D    246
SON1   AATAGAAGAAAATTGTTTGCAACAAGAGCAACACCAAGAGGAGCCTTTACTGTCATTGGA    737

Son1p  V  T  P  I  S  M  F  G  S  D  Q  K  T  G  R  A  K  S  S  S    266
SON1   TGTTACACCAATCTCAATGTTTGGCTCAGATCAAAAAACGGGTCGTGCCAAGAGTTCTAG    797

Son1p  H  L  F  N  E  Y  S  Y  V  D  S  N  M  D  S  I  S  S  V  V    286
SON1   TCATTTATTTAATGAGTACAGTTACGTTGACTCTAACATGGACAGCATTTCCAGTGTTGT    857

Son1p  S  E  D  L  L  D  E  R  G  H  E  K  I  E  D  E  D  E  D  N    306
SON1   ATCTGAAGATCTGTTAGATGAACGGGGACATGAGAAGATAGAGGATGAGGATGAGGATAA    917
              g
Son1p  D  L  D  E  D  D  I  Y  D  I  S  L  L  K  N  R  R  K  Q  S    326
SON1   TGATCTTGATGAAGACGATATCTACGATATCTCTCTCTTGAAGAACAGAAGAAAGCAAAG    977
                         r        r
Son1p  F  V  L  N  K  N  T  I  D  F  E  R  F  P  S  P  S  T  S  A    346
SON1   TTTTGTCCTCAATAAAAACACTATTGATTTTGAAAGATTTCCATCTCCCTCAACCTCGGC   1037

Son1p  N  V  P  S  T  A  T  T  G  K  R  K  P  A  K  S  S  S  N  R    366
SON1   AAACGTACCGTCTACTGCTACTACCGGTAAAAGGAAACCAGCAAAATCATCCAGTAACCG   1097
                                                  *  *  *  *  *
Son1p  S  C  V  S  N  S  N  E  N  G  T  L  E  R  I  K  K  P  T  S    386
SON1   TAGTTGCGTTAGTAACAGTAATGAAAACGGCACATTAGAAAGAATAAAGAAGCCTACATC   1157
       *  *  *  *  *  *  *  *  *  *  *  *
Son1p  A  V  V  S  S  N  A  S  R  R  K  L  I  N  Y  T  K  K  H  L    406
SON1   AGCTGTAGTAAGCTCAAATGCTAGTAGGCGGAAGCTAATTAATTATACTAAGAAGCACTT   1217
        p
Son1p  S  S  H  S  S  T  N  S  N  S  K  P  S  T  A  S  P  S  A  H    426
SON1   ATCTTCACATTCATCTACAAATTCGAATTCGAAACCTTCGACTGCATCACCATCGGCCCA   1277
                                 e
Son1p  T  S  S  S  D  G  N  N  E  I  F  T  C  Q  I  M  N  L  I  T    446
SON1   TACGTCATCTTCTGACGGTAATAACGAAATATTTACGTGTCAGATAATGAATCTCATTAC   1337

Son1p  N  E  P  C  G  A  Q  F  S  R  S  Y  D  L  T  R  H  Q  N  T    466
SON1   AAATGAACCGTGTGGTGCCCAATTTTCAAGGTCCTATGATTTAACGAGACACCAAAATAC   1397

Son1p  I  H  A  K  R  K  I  V  F  R  C  S  E  C  I  K  I  L  G  S    486
SON1   CATTCACGCTAAAAGGAAGATTGTCTTCCGTTGCTCGGAGTGTATAAAAATTCTTGGATC   1457

Son1p  E  G  Y  Q  K  T  F  S  R  L  D  A  L  T  R  H  I  K  S  K    506
SON1   TGAGGGCTATCAGAAGACGTTTTCGAGACTGGATGCTTTAACAAGGCATATAAAATCGAA   1517

Son1p  H  E  D  L  S  L  E  Q  R  Q  E  V  T  K  F  A  K  A  N  I    526
SON1   GCATGAAGATTTGTCGTTAGAACAACGTCAAGAAGTTACAAAATTTGCAAAGGCTAATAT   1577

Son1p  G  Y  V  M  G  *  531
SON1   TGGTTATGTCATGGGTTAATTAAGGTTACAAATTATATAGGAGATAAAAGAAGAAAACCT   1637

SON1   CACACAAAATGGAATACATATACACATCTATATATATTTACAATATATATCATATATCTG   1697

SON1   CATTCAGGAATGTTCTTATAAATATCACATTTTTAAAGTACCTCATTGGACTTATAATAA   1757
                                        d
SON1   GTTTTCTATTGGTTTTCATTATACTTCGGAAAATACACAATTATATTATATACTTACCCC   1817

SON1   CCTTAAGGATTTTTTATGAAACCCTCATTACGGACTTTCTCAGCATTGATCTTAGCAGAT   1877

SON1   TCAGGATCTAGGTAGAATTTTCTGATAAACTTAAGACCATTATTCTTATCTAATTCAACG   1937

SON1   ACTAAGGGGATACCATTTGGAATATCAACATTCTTGATGTCATCATCTGATATACCCTCC   1997

SON1   AGAATTTTCAGTAGCGATCTCACTGAACTTCCATGG  2033
                                       n
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FIGURE 5.—Nucleotide sequence of *SON1* and its flanking regions. The translated Son1p protein sequence is shown above the DNA sequence. Asterisks mark the putative bipartite NLS. Amino acids in the two acidic domains are underlined. Lowercase letters below underlined nucleotides are used to mark the following restriction enzyme recognition sites: b, *Bam*HI; d, *Dra*I; e, *Eco*RI; g, *Bgl*II; n, *Nco*I; P, *Pvu*II; r, *Eco*RV; s, *Sma*I; x, *Xba*I. The sequence of nucleotides (−361) to (+1750) has been confirmed by analysis of both DNA strands.

son1::LEU2 haploid, PSY316, could also be directly obtained by transformation, *SON1* must not be an essential gene. PSY316 grows well at 36° and 30° but, like *son1-1*, *son1-2* and *son1-3* strains, is slower growing at 23° and 14°. As described above, *SEC63 son1::LEU2* cells also show a partial defect in nuclear protein localization, as judged by indirect immunofluorescence (Figure 3).

A *son1::URA3* allele (PSY323) was created by direct integration of a *SON1/URA3* fragment from pMN70 (identical to pMN69 except that *URA3* replaces *LEU2*) into W303$\alpha\Delta$*cyt1*. Integration was confirmed by Southern analysis (data not shown). PSY323 was used to assess the effect of *son1::URA3* on the localization of an NLS-cytochrome c_1 fusion protein (Table 6; see above).

In order to determine what effect a *son1* null allele has on the Ts⁻ defect of *sec63-101*, PSY316 (*SEC63 son1::LEU2*) was crossed to *sec63-101*. In 32 tetrads derived from the resulting diploid, only 8 showed 2:2 segregation of the Ts⁻ phenotype. Of the remaining tetrads, 4 were scored as nonparental ditype (4 Ts⁺:0 Ts⁻) and 20 were scored as tetratype (3 Ts⁺:1 Leu⁻ Ts⁻). Thus, *son1::LEU2* can suppress the Ts⁻ phenotype of *sec63-101*. As judged by colony size at 36°, the degree of suppression by *son1::LEU2* is comparable to that observed for *son1-1*, *son1-2* and *son1-3*.

Since the *son1-1* mutant suppresses the Ts⁻ defect of *sec63-101* but not of *sec63-1*, we determined if the *son1* null allele can also distinguish between the two *sec63* alleles. For all 18 complete tetrads obtained in a cross between PSY320 (*SEC63 son1::LEU2*) and *sec63-1*, all showed 2:2 segregation of the Ts⁻ phenotype. Thus, like the *son1-1* allele, *son1::LEU2* suppresses the Ts⁻ defect of *sec63-101* but not that of *sec63-1*.

Secretory protein translocation in *son1* strains: *sec63-1* mutants are defective in ER protein translocation (ROTHBLATT *et al.* 1989). We asked whether *son1* mutations affect processing of secretory proteins. The lumenal ER protein Kar2p has a single precursor that accumulates in early *sec* mutants (ROSE, MISRA and VOGEL 1989). We used immunoprecipitation of steady-state labeled cell extracts to compare preKar2p and Kar2p levels in *sec63-1* (RSY151), *son1-1*, *son1::LEU2*, and wild-type cells grown at either 23° or 30°. As predicted, pre-Kar2p accumulates in *sec63-1* cells. No accumulation of pre-Kar2p was visible in *son1* mutants (data not shown).

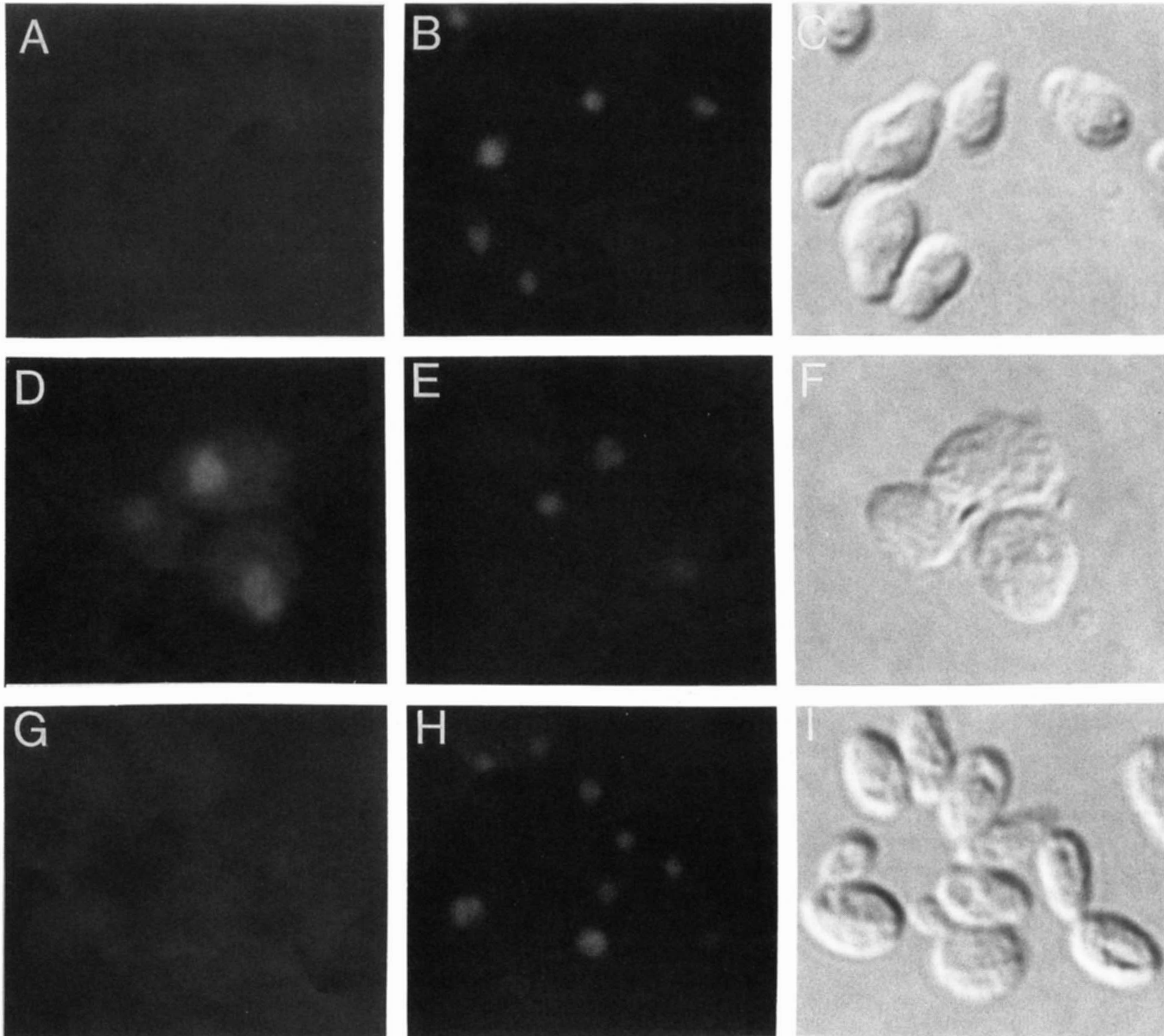

FIGURE 6.—Son1p-invertase is nuclear-localized. W303α*Δcyt1* transformants were grown in selective media at 30°, fixed and prepared for indirect immunofluorescence. (A–C) pMN94 (P_{GAL1}-*SON1-SUC2*) transformants grown in 5% raffinose. (D–F) pMN94 transformants following 6 hr of galactose induction. (G–I) YEp352 transformants after 6 hr of galactose induction. Panels A, D and G show cells treated with anti-invertase antibody and FITC-conjugated goat anti-rabbit IgG. DAPI staining was used to visualize DNA (B, E, and H). Cells viewed by Nomarski optics are shown in panels C, F and I.

DISCUSSION

Sec63p of *S. cerevisiae* plays an essential role in translocation of proteins into the ER, as well as a role in maintenance of nuclear integrity. Sec63p contains three membrane-spanning regions, a short ER-lumenal domain with similarity to the *E. coli* heat-shock protein DnaJ, and a large cytoplasmic domain. To better understand the functions of different parts of Sec63p, we sequenced several Ts^- mutations in *SEC63* and isolated mutations that suppress *sec63-101*. The recessive extragenic suppressors define five genes, *SON1–SON5*. The *SON1* gene encodes a protein of 531 aa that is not similar to other known proteins. Deletion of *SON1* suppresses the Ts^--lethal phenotype of *sec63-101* but causes a small, but detectable, defect in localization of nuclear proteins. A Son1p-invertase fusion protein is localized to the yeast nucleus.

Sec63p structure and function: Both genetic and biochemical analyses suggest that Sec63p is involved in ER protein translocation. *sec63-1* mutants accumulate membrane-associated secretory intermediates (ROTHBLATT *et al.* 1989). Microsomes isolated from *sec63-1* and *ptl1* mutants are defective in *in vitro* translocation assays (ROTHBLATT *et al.* 1989; TOYN *et al.* 1988). Sec61p and Sec62p can be cross-linked to Sec63p (DESHAIES *et al.* 1991). In addition, *sec61-2* and *sec62-1* show synthetic lethality in combination with *sec63-1* (ROTHBLATT *et al.* 1989). Hence, these three proteins are proposed to be part of a multisubunit translocation complex.

Several pieces of genetic data, however, imply that

Sec63p also affects localization of nuclear proteins. First, three different mutations in *SEC63* (*sec63-101*, *sec63-106* and *sec63-107*) were recovered in two different screens for *npl* mutants (SADLER *et al.* 1989; BOSSIE *et al.* 1992). Second, *sec63-101* mutants show little, if any, defect in ER translocation (SADLER *et al.* 1989). Third, *son1* mutations, isolated on the basis of their ability to suppress *sec63-101*, cause a partial defect in nuclear protein localization. All of the *npl* class of *sec63* alleles (*e.g.*, *sec63-101*) are found in the C-terminal cytoplasmic domain of the protein, while the severely secretion-defective *sec63-1* allele affects the DnaJ-homologous region of the protein, located in the ER lumen. *son1* mutations cannot suppress either the *sec63-1* mutation or mutations in other ER translocation mutants such as *sec61-2*, *sec62-1* or *kar2-159*. ER translocation, at least in the case of Kar2p, is unaffected in *son1* mutants. *sec63* mutations do not form a simple allelic series: *sec63-1* is stronger than *sec63-101* in that it profoundly affects secretion, but weaker in that it is lethal only at 38°.

The nuclear localization defect in *sec63* mutants appears to be due to a failure to retain proteins in the nucleus (A. CHIANG and P. SILVER, unpublished results). In contrast, *npl3* and *npl6* mutants show normal retention of nuclear proteins and seem to interfere with the process of protein import (BOSSIE *et al.* 1992; A. CHIANG and P. SILVER, unpublished results). Since nuclear antigens are correctly localized in *sec62-1* cells (SADLER *et al.* 1989), it is unlikely that the Npl$^-$ phenotype is a general property of early *sec* mutants.

Characteristics of *son* mutations: Of 1625 Ts$^+$ pseudorevertants of the *sec63-101* mutant, mutations in 1258 are dominant, and may all be intragenic. The other 367 are recessive and are extragenic suppressor mutations. Fifty-eight of the 367 fall into four complementation groups; the remaining recessive mutants remain largely uncharacterized.

The *son* mutations show different spectra of suppression for *sec63* phenotypes. The *sec63-101* mutant was originally isolated because it showed some mislocalization of an NLS-cytochrome c_1 fusion protein at 30° and failed to grow at 36° (SADLER *et al.* 1989). In the appropriate *cyt1* background, the mislocalization of the NLS fusion protein from the nucleus to the mitochondria is detected by growth on glycerol. Cells are Gly$^+$ even under conditions where they are completely viable and show a relatively weak defect in localization of nuclear antigens as judged by indirect immunofluorescence. Thus, the ability to grow on glycerol as the sole carbon source is an extremely sensitive assay for defects in nuclear protein localization. Mutations in *SON1* and *SON2* suppress only the Ts$^-$ lethality caused by *sec63-101* (and other mutations in the C terminus of Sec63p). Mutations in *SON4*, however, suppress both the glycerol-growth phenotype of *sec63-101* and the Ts$^-$ lethality caused by *sec63-1*, suggesting that, in this regard, *son4* is a stronger suppressor than *son1* or *son2*.

The Son1p protein: The sequence of the *SON1* gene reveals a 531 codon open reading frame encoding a novel protein. Son1p contains two stretches of mixed acidic residues, similar to the one found at the extreme C terminus of Sec63p. Son1p also contains a bipartite NLS consensus (DINGWALL and LASKEY 1991; ROBBINS *et al.* 1991).

Son1p activity is required for normal growth at low temperatures. The ability of *son1* strains to grow normally at higher temperatures may indicate that another protein, with overlapping function, is active only at temperatures above 23°. In Southern blots probed with *SON1*, additional bands, unaffected by the integrity of the chromosomal *SON1* gene, are sometimes visible (data not shown). These bands could be produced by cross-hybridization with a related gene. Alternatively, Son1p may perform a function that is required only at low temperatures. For example, Son1p could be necessary for stimulation of a process that becomes increasingly inefficient as growth temperature decreases.

As judged by indirect immunofluorescence, Son1p influences the net distribution of nuclear antigens. It is not clear, however, that Son1p participates directly in the process of nuclear protein import. Most *son1* mutants do not missort NLS-cytochrome c_1 to the mitochondria. Furthermore, only a limited degree of mislocalization of SV40-invertase and histone H2B is observed. This partial effect may be due to the existence of proteins with overlapping functions. Or, Son1p, like Sec63p, may play a role in nuclear integrity. The aberrant morphology of *son1* nuclei is consistent with the latter hypothesis. We have not directly assayed the effect of *son1* mutations on retention of nuclear proteins.

Deletion of *SON1* is not lethal and can suppress the *sec63-101* mutation. In a formal sense, this result implies that Son1p is toxic in a *sec63-101* mutant at the nonpermissive temperature. When expressing Son1p at very high levels (from a galactose-inducible gene on a multicopy plasmid), both wild-type and *sec63-101* cells show a reduced rate of growth, even at 23° (data not shown). Suppression via elimination of Son1p activity is consistent with the high frequency at which *son1* mutants were observed among the recessive suppressors. The inability of either *son1-1* or *son1::LEU2* to suppress the Ts$^-$ phenotype of *sec63-1* suggests that *sec63-101* and *sec63-1* mutants are sensitive to growth at 36° for different reasons.

son1-1 does not suppress *sec63::URA3*. Thus, a *son1* mutation appears capable of suppressing only partial loss of Sec63p function. Son1p might, for example, inhibit the function of Sec63p. In immunoblots using

anti-Sec63p antiserum, no significant difference in Sec63p size or levels was noted between *sec63-101 SON1* and *sec63-101 son1* mutants or between wild-type and *son1* cells (data not shown). Hence, Son1p inhibition of Sec63p, if it occurs, probably does not take place at the transcriptional or translational level. Alternatively, there may be a target protein that interacts with the acidic C terminus of Sec63p and, when Sec63p function is reduced, Son1p could, through its acidic regions, interact with the target in a nonproductive manner. This interaction between Son1p and the hypothetical target could be fortuitous, or it could be a mechanism by which Son1p normally regulates Sec63p function.

A Son1p-invertase fusion protein is largely localized to the yeast nucleus, suggesting that Son1p itself is also predominantly or entirely a nuclear protein. If Sec63p resides in the inner nuclear membrane, as well as in the nuclear envelope/ER network, then Son1p and the C-terminal domain of Sec63p would both be in contact with the nucleoplasm. This is consistent with the idea that Son1p and the C terminus of Sec63p together play a role in nuclear structure or function. Characterization of other *SON* genes will be useful in developing more specific models for the role of the C terminus of Sec63p.

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