

A Cytoskeleton-related Gene, *USO1*, Is Required for Intracellular Protein Transport in *Saccharomyces cerevisiae*

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Abstract. The *Saccharomyces cerevisiae* mutant strains blocked in the protein secretion pathway are not able to induce sexual aggregation. We have utilized the defect of aggregation to concentrate the secretion-deficient cells and identified a new gene which functions in the process of intracellular protein transport. The new mutant, *usol*, is temperature sensitive for growth and protein secretion. At the restrictive temperature (37°C), *usol* mutant accumulated the core-glycosylated precursor form of the exported protein invertase in the cells. Ultrastructural study of the mutant fixed by the freeze-substitution method revealed expansion of the nuclear envelope lumen and

accumulation of the ER at the restrictive temperature. Abnormally oriented bundles of microtubules were often found in the nucleus. The *USO1* gene was cloned by complementation of the *usol* temperature-sensitive growth defect. DNA sequence analysis revealed a hydrophilic protein of 1790 amino acids with a COOH-terminal 1,100-amino acid-long α -helical structure characteristic of the coiled-coil rod region of the cytoskeleton-related proteins. These observations suggest that *Usol* protein plays a role as a cytoskeletal component in the protein transport from the ER to the later secretory compartments.

IN the eukaryotic cells, proteins destined for the extracellular environment, plasma membrane, or lysosomes are first synthesized as precursor polypeptides in the cytoplasm. They are translocated across the ER membrane and transported along the membrane-enclosed organelles. Sorting for their final destinations and various processings and modifications occur during the process. Finally, they reach the ultimate site of residence and express their full function (Palade, 1975).

The secretory process in *S. cerevisiae* has been successfully studied by genetic approaches. First, Schekman and co-workers isolated the series of temperature-sensitive secretion mutants (*sec1-23,53*, and *59*) by utilizing the density increase of the cells that accumulate secretory proteins (Novick et al., 1980; Ferro-Novick et al., 1984). By the accumulation of secretory organelles and the modification of the accumulated proteins at nonpermissive temperature, the *sec* mutants have been classified in several groups with distinct functions in the secretory pathway: (a) protein translocation across the ER membrane; (b) transport from the ER to the Golgi apparatus; (c) transit through the Golgi apparatus to the secretory vesicles; and (d) exocytosis (Novick et al., 1981).

Additional mutant strains in the process of translocation

into the ER, *sec61,62,63*, *ptll*, and *2* have been isolated by utilizing the mislocalization to the cytoplasm of prepro- α -factor-*HIS4* or prepro- α -factor-*TRP1* fusion proteins (Deshaies and Schekman, 1987; Deshaies et al., 1988a; Rothblatt et al., 1989; Toyn et al., 1988). Mannose suicide selection was utilized to isolate additional ER-Golgi transport mutants, *bet1* and *bet2* (Newman and Ferro-Novick, 1987).

Protein transport from the ER to the Golgi is the first step of intercompartmental transfer, and requires more than 14 genes: *SEC12,13,16,17,18,20,21,22,23*, *BET1,2*, *SARI*, *YPT1*, and *ARF1* (Novick et al., 1980; Newman and Ferro-Novick, 1987; Nakano and Muramatsu, 1989; Segev et al., 1988; Stearns et al., 1990). Temperature-sensitive mutants of these genes show exaggeration of the ER-like membrane structures and accumulation of the core-glycosylated form of secretory and vacuolar proteins at the restrictive temperature. Some of the gene products have been characterized. *Sec12p* is an integral membrane protein, and *Sec23p* is peripherally associated with the cytoplasmic face of membranes (Nakano et al., 1988; Hicke and Schekman, 1989). Predicted sequences of *Sarlp* and *Yptlp* show the characteristics of GTP-binding proteins (Nakano and Muramatsu, 1989; Gallwitz et al., 1983). *Sec18p* is reported to be the yeast homologue of NSF (*N*-ethylmaleimide-sensitive fusion protein), a protein factor required for transport from the ER to the Golgi in vivo and in vitro (Eakle et al., 1988). *Sec17p* is reported to be one of the yeast homologues of soluble NSF attachment protein (SNAP), which enables NSF to bind to the Golgi membranes (Clary et al., 1990).

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Analysis of a mutation of *YPT1* gene suggested a role in microtubule organization, and that of another *YPT1* mutant allele showed that protein transport was mainly blocked in the Golgi (Schmitt et al., 1986, 1988). Cytoplasmic and intranuclear microtubules are necessary for migration and proper orientation of the nucleus, spindle pole bodies (SPBs)¹ separation, and nuclear division. Recent experiments utilizing microtubular depolymerizing antibiotics have shown that microtubules seem to be involved in some of the intracellular protein transport steps accompanied by membrane traffics (Kelly, 1990). Kinesin is reported as one of the driving motors of vesicle transport along the microtubules. Kinesin heavy chain has three domains; the head domain has ATP-dependent microtubule binding activity; the rod domain forms α -helical coiled-coil structure; and the fan-shaped domain links the vesicles (Hirokawa et al., 1989; Yang et al., 1989).

Actin is the other major component of cytoskeleton and has essential roles in cytokinesis, chromosome segregation, and organellar transport. In *S. cerevisiae*, actin microfilaments organize along the long axis of cells. Conditional lethal actin mutant, *act1*, exhibits partial inhibition of secretion of the periplasmic protein and intracellular accumulation of secretory vesicles (Novick and Botstein, 1985). *SEC2* is required at the final stage of the secretory pathway; the amino terminal region of Sec2p is in an α -helical, coiled-coil conformation and is essential for vesicular transport (Nair et al., 1990). Transport of proteins from the ER to the Golgi stack and between the cisternal compartments of the Golgi is mediated by vesicular carriers. To transport the vesicles efficiently between the secretory organelles, driving mechanisms and intracellular frameworks may play an essential function. But almost nothing is known about the motor molecules and the cytoskeleton components involved in protein transport from the ER to the Golgi apparatus.

We have isolated a novel temperature-sensitive secretory mutant which is blocked in protein transport from the ER to the Golgi, and named it *usol*. Observation of intracellular fine structure of the *usol* cells and genetic analysis of the *USO1* gene suggest that *USO1* encodes a cytoskeleton-related molecule which has an essential role in protein transport from the ER to the Golgi.

Materials and Methods

Strains and Culture Conditions

E. coli strains used in this study were MC1061 (F⁻ *hsdR mcrB araD* Δ [*ara-leu*] Δ *lac galU galK rpsL thi*) (Casadaban and Cohen, 1980), and MV1184 (*ara* Δ [*lac-proAB*] *rspL thi* [Φ 80 *lacZ* Δ M15] Δ [*srl-recA*] 306::Tn10[*tet*]^r F' [*traD36 proAB lacI^q lacZ* Δ M15]) (purchased from Takara Shuzo Co., LTD., Kyoto, Japan) for cloning experiments and nucleotide sequencing. Helper phage M13K07 for single-stranded DNA preparation (Vieira and Messing, 1987) was also from Takara Shuzo Co., LTD. The genotypes and sources of the strains of the *S. cerevisiae* used in this study are listed in Table 1.

Yeast strains were usually grown at 30°C in YEP2D medium (1% yeast extract, 2% polypeptone, 2% glucose) or in SD medium (0.67% Bacto-yeast nitrogen base without amino acids, 2% glucose, and appropriate supplements). Invertase repression medium, YEP7D, contained 7% glucose, and invertase-inducing medium, YEP2S, contained 2% sucrose instead of glucose. Temperature-sensitive *sec* and *usol* strains were cultured at 25°C and incubated at 37°C for restrictive experiments.

1. *Abbreviations used in this paper:* SPB, spindle pole body; ORF, open reading frame.

Plasmid DNA Manipulations

E. coli plasmids, pUC118 and pUC119 (Vieira and Messing, 1987); yeast single-copy plasmid YCpG11 (Ohya et al., 1986); yeast multi-copy plasmid pHN114 (1.45-kb EcoRI fragment of YRp7 [Struhl et al., 1979] containing the *TRP1-ARS1* gene was inserted into the unique BbeI site of pUC19 [Yanisch-Perron et al., 1985] to utilize α -complementation system on yeast vector); and yeast integration plasmid YIp5 (Botstein and Davis, 1982) have been described elsewhere. Deletion mapping and subcloning analysis were performed by using multi-copy plasmid YRp7 and pHN114. DNA manipulations including plasmid DNA isolations, restriction enzyme digestions, ligations, and *E. coli* transformations were carried out by the standard methods (Maniatis et al., 1982). The 4.5-kb SmaI-XhoI fragment of pHN155 showed a complementing activity of the *usol* mutation. A series of successive deletions in subcloning analysis were performed by using Kilo-Sequence Deletion Kit (Takara Shuzo Co., Ltd.) (Henikoff, 1984). The 9.5-kb BamHI-SalI fragment containing the full length of the cloned yeast DNA, which has the *usol* complementation activity, was inserted into the multicloning sites of pHN114. In the first deletion, KpnI site in the vector and SmaI site in the cloned DNA fragment were cleaved and successive deletion by Exonuclease III digestion was only progressed from the SmaI site. Deletion series plasmids were prepared at intervals of 200–300 bp and then transformed into the strain USO1-10 (a, *usol trp1*). The transformants were assayed for the restoration of growth at the restrictive temperature. The smallest plasmid (Δ 17) which has a complementation activity was subjected to the second deletion. SphI site in the vector and XhoI site in the cloned DNA were cleaved to perform Exonuclease III digestion only from the XhoI site, and assayed by transformation into the strain USO1-10. The plasmid Δ 45 had the smallest DNA fragment, 3.3 kb, containing the complementation activity. The first and the second deletion series plasmids were utilized for determination of DNA sequences.

DNA nucleotide sequences were determined by the dideoxy method (Sanger et al., 1977) and DNA sequencing system (model 370A; Applied Biosystems, California). Yeast cells were transformed to Ura⁺ or Trp⁺, with plasmids or linear DNA, on minimal SD media by the lithium acetate method (Ito et al., 1983).

Isolation of Temperature-sensitive *usol* Mutant

Yeast strain K382-19D was mated with DBY747 and sporulated to select a host strain, which had appropriate auxotrophic marker and recessive antibiotics resistant markers and showed high efficiency of sexual agglutination. Resultant strain HKD2 cells grown in YEP2D medium ($\sim 2 \times 10^8$ cells) were mutagenized with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (100 μ g/ml) in 0.1 M malate buffer (pH 5.2) at 25°C for 30 min and then incubated in YEP2D medium at 25°C for 25 h to fix the mutation. 2-mercaptoethanol was added (final concentration, 1.0%) to remove the constitutively expressed agglutinin from the cell surface and incubated for 2 h. Cells were washed three times with 10 mM Tris-HCl (pH 8.0) and incubated in fresh YEP2D medium containing 2 μ M synthetic α -factor (Bachem Feinchemikalien AG, Bubendorf, Switzerland) at 37°C for 3 h to induce the sexual agglutinin. The A5-8-1C (α type) cells were added in the culture. The mixture was slightly spun down and incubated at 37°C for 10 min to make sexual agglutination. The pelleted cells were resuspended by gentle agitation and set to stand for 5 min to sediment the agglutinated cells. The floating cells were collected and spread on YEPD plate containing cycloheximide (1 μ g/ml), which inhibits the growth of α type and a/ α diploid cells, and incubated at 25°C for 4–5 d. To select temperature-sensitive mutant strain, colonies were replica plated onto YEP2D medium containing cycloheximide and incubated at 37°C. The colonies with Ts growth were replicated from the master plates onto phosphate-free minimal medium to derepress the synthesis of acid phosphatase, and after 5 h incubation at 25 and 37°C the replicas were stained for secreted acid phosphatase (Hansche et al., 1978). The clones that showed Ts secretion of acid phosphatase were screened for conditional defect in secretion and glycosylation of invertase by nondenaturing gel electrophoretic analysis. 20 clones were selected as candidates of temperature-sensitive secretion or glycosylation mutants.

Nondenaturing Gel Electrophoretic Analysis of Invertase

External and internal invertase were analyzed on nondenaturing polyacrylamide gels as described by Kaiser et al. (1987) with slight modifications. *usol* (USO1-3), *sec18* (S18-1), and wild type (X2180-1A) cells were grown in 10 ml YEP7D medium at 25°C (10^8 cells), transferred to 10 ml YEP2S

Table I. Yeast Strains

Strains	Genotype	Source
X2180-1A	a <i>SUC2 CUP1</i>	a
DBY747	a <i>ura3 trp1 leu2 his3</i>	a
S18-1	α <i>sec18 ura3</i>	b
K382-19D	α <i>spo11 ura3 can1 cyh2 ade2 his7 hom3 tyr1</i>	c
HKD2	a <i>ura3 trp1 cyh2 leu2 ade</i>	
ts1935	a <i>uso1-1 ura3 trp1 cyh2 leu2 ade</i>	
A5-8-1A	a <i>leu1</i>	a
A5-8-1C	α <i>leu1</i>	a
USO1-3	a <i>uso1-1 ura3 leu1 leu2</i>	
USO1-10	a <i>uso1-1 ura3 leu2 trp1</i>	
USO1-24	α <i>uso1-1 ura3 leu1 trp1 ade2 his7</i>	
DBY747-U	a <i>USO1::URA3 ura3 leu2 trp1 his3</i>	
DBY747-U	a <i>USO1::URA3 ura3 LEU1 leu2 trp1 HIS7 his3 ADE2</i>	
USO1-24	α <i>uso1-1 ura3 leu1 LEU2 trp1 his7 HIS3 ade2</i>	
DBY747	a <i>USO1 ura3 LEU1 leu2 trp1 HIS7 his3 ADE2</i>	
USO1-24	α <i>uso1-1 ura3 leu1 LEU2 trp1 his7 HIS3 ade2</i>	
ΔUSO1-3	a <i>USO1 ura3 LEU1 leu2 trp1 HIS7 his3 ADE2</i>	
	α <i>Δuso1-1::URA3 ura3 leu1 LEU2 trp1 his7 HIS3 ade2</i>	
ΔUSO1-4	a <i>Δuso1::URA3 ura3 LEU1 leu2 TRP1 HIS7 his3 ADE2</i>	
	α <i>uso1-1 ura3 leu1 LEU2 trp1 his7 HIS3 ade2</i>	
Strains for Complementation		
HS1	α <i>sec1 leu1</i>	b
HS2	α <i>sec2</i>	b
HS3	α <i>sec3</i>	b
HS4	α <i>sec4 leu1</i>	b
HS5	α <i>sec5 leu1</i>	b
HS6	α <i>sec6 leu1</i>	b
HS7	α <i>sec7 leu1</i>	b
HS8	α <i>sec8</i>	b
HS9	α <i>sec9 ura3 cyh2</i>	b
HS10	α <i>sec10 ura3 cyh2</i>	b
HS11	α <i>sec11 leu1</i>	b
HS12	α <i>sec12 leu1</i>	b
HS13	α <i>sec13 ura3 cyh2</i>	b
HS14	α <i>sec14 leu1</i>	b
HS15	α <i>sec15 ura3 cyh2</i>	b
HS16	α <i>sec16 ura3 cyh2</i>	b
HS17	α <i>sec17 ura3 cyh2</i>	b
HS18	α <i>sec18 leu1</i>	b
HS19	α <i>sec19 leu1</i>	b
HS20	α <i>sec20 leu1</i>	b
HS21	α <i>sec21 leu1</i>	b
HS22	α <i>sec22 leu1</i>	b
HS23	α <i>sec23 leu1</i>	b
HS53	α <i>sec53 leu1</i>	d
HS59	α <i>sec59 leu1</i>	d

Reference or source: (a) yeast genetic stock center; (b) Novick et al. (1980); (c) Klapholz and Esposito (1980); (d) Ferro-Novick et al. (1984); others, this study.

medium and further incubated at 37°C. Cells of 1.0 ml culture were collected by centrifugation, washed once and incubated in 200 μl of 10 mM Tris-HCl (pH 8.0) containing 1.4 M sorbitol, 0.2% 2-mercaptethanol, 10 mM Na₃N, and 2 U of Zymolyase 100,000, at 30°C for 30 min. Spheroplasts and released cell surface fraction were separated by centrifugation and spheroplasts were lysed with 10 mM Tris-HCl buffer containing 0.2% Triton X-100. Crude extracts were heated to 50°C for 10 min before loading onto 6.75% nondenaturing polyacrylamide gels, in order to dissociate multimeric enzyme to a monomeric form. Invertase was localized in gels after incubation in 0.2 M sodium acetate (pH 4.8) containing 0.2 M sucrose for 1 h at 30°C and stained with 0.1% 2,3,5-triphenyltetrazolium chloride in

0.1 N NaOH (Goldstein and Lampen, 1979). Invertase activity was quantitated by measuring the production of reducing sugar from sucrose using 3,5-dinitrosalicylic acid (Bernfeld, 1955).

Fluorescent Microscopy

uso1 and wild type cells were grown in YEP2D medium at 25°C and then at 37°C for 1 h. The cells were washed with 0.2 M potassium phosphate (pH 8.2) and incubated with 20 mM chloroquine in 0.2 M potassium phosphate (pH 7.5) containing 0.2% glucose at 37°C for 45 min (Lenz and Holzer, 1984). Fluorescence observations were made with an Olympus Vanox

AH2-FL with a UVFL 100× objective lens and an ultraviolet excitation filter.

Freeze-substituted Fixation Method of EM

Preparation of thin sections of yeast cells by freeze-substitution method was carried out as described (Kanbe and Tanaka, 1989) with minor modifications. Cells grown at 25°C and then incubated at 37°C for 2 and 7 h were collected by centrifugation. Pellets of the cells were mounted on the copper meshes to form thin layer and plunged into liquid propane cooled with liquid N₂. Frozen cells were transferred to 2% OsO₄ in anhydrous acetone, and kept at -80°C for 48 h with solid CO₂/acetone. They were transferred to -35°C for 2 h, 4°C for 2 h, and then to room temperature for 2 h. After washing with absolute acetone three times, samples were infiltrated with increasing concentrations of Spurr's resin in absolute acetone and finally with 100% Spurr's resin. They were then polymerized in capsules at 50°C for 5 h and 70°C for 30 h. Thin sections cut with Sorvall MT-2 ultramicrotome were collected, stained with uranyl acetate and lead citrate, and observed in a JEOL 200 CX electron microscope at 100 kV.

Results

Isolation of *usol* Mutant

To isolate new secretion mutants, we applied a new method for enrichment of secretory mutant cells.

S. cerevisiae haploid cells have two mating types, *a* and α . One of the fastest events that occurs when *a* and α cells are mixed is cellular agglutination. This process is mediated by cell surface glycoproteins called *a*- and α -agglutinin, which are expressed on *a* and α cells, respectively (Sprague et al., 1983). These agglutinins are present at a low level on the cell surface constitutively, and are induced by exposure to the mating pheromone secreted by the opposite mating type cells (Terrance and Lipke, 1987). The induced agglutinins are exported to the cell surface through the secretion pathway and function to increase agglutinability. Therefore, the mutant strains which are blocked in the secretory pathway are expected not to induce the sexual agglutination.

To isolate novel conditional secretion mutants, HKD2 (*a*, *cyh2*) cells were subjected to mutagenesis with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (3–5% survival). After incubation at 25°C for 25 h for fixation of mutations, the cells were treated with 2-mercaptoethanol to remove the preexisting cell surface agglutinin (Orlean et al., 1986), and then induced for the sexual agglutinin by treatment with the synthetic α -factor at 37°C for 3 h. By addition of excess α type cells, normal cells performed sexual agglutination and sedimented. Then floating cells were collected, plated, and incubated at 25°C for 4–5 d. Colonies were replicated onto two plates and incubated at 25°C or 37°C for 3 d. Strains that were Ts⁻ for growth were obtained at high frequency (5–10%) by this selection. Of the 2,990 colonies that showed Ts⁻ growth, 396 strains were found Ts⁻ for secretion of acid phosphatase. To categorize these Ts⁻ mutations into translocation, transport, or glycosylation defects, the mutant strains were assayed for their synthesis and secretion of invertase by nondenaturing gel electrophoretic analysis (Goldstein and Lampen, 1979). It was found that 20 of the Ts⁻ mutants accumulated abnormally glycosylated invertase in the cells at the restrictive temperature. Five strains, which clearly showed secretion defect, were subjected to complementation analysis with 25 previously reported secretion mutants (*sec23*, *sec53*, and *sec59*). Three strains were identified to be allelic to *sec7*, *sec23*, and *sec53*, and two strains were able to complement all of the *sec* mutants. One of them, ts1935, showed a

stringent block of the enzyme secretion and rapidly accumulated incompletely glycosylated invertase in the cells at the restrictive temperature. To determine whether the observed phenotype was due to a single or multiple mutations, ts1935 was crossed to wild type cells; resulting diploid cells did not show the temperature-sensitive defects. Tetrad analysis of this diploid strain showed 2:2 cosegregation of temperature-sensitive defects of growth and invertase secretion (data not shown). These observations indicated that ts1935 mutation is a recessive single mutation on a chromosomal gene. We defined the new gene as *usol* (*YUSOU*; meaning transport in Japanese), which functions in intracellular protein transport.

usol Mutant Accumulates Core-glycosylated Invertase

A failure to export proteins to the cell surface often results in accumulation of specific forms of protein precursors in a distinct organelle. To investigate the stage of the secretion block, invertase induction and secretion were analyzed in *usol* cells. External invertase is derepressed for synthesis by depletion of glucose in the medium (Carlson and Botstein, 1982), and in the wild type, is transported to the cell surface along the secretory pathway within 5 min (Novick et al., 1981).

usol, *sec18*, and wild type cells were grown at the permissive temperature (25°C) in YEP7D medium and transferred to the restrictive temperature (37°C) in YEP2S medium. Samples were collected at appropriate time intervals. The yeast cell wall was enzymatically lysed to generate sphero-

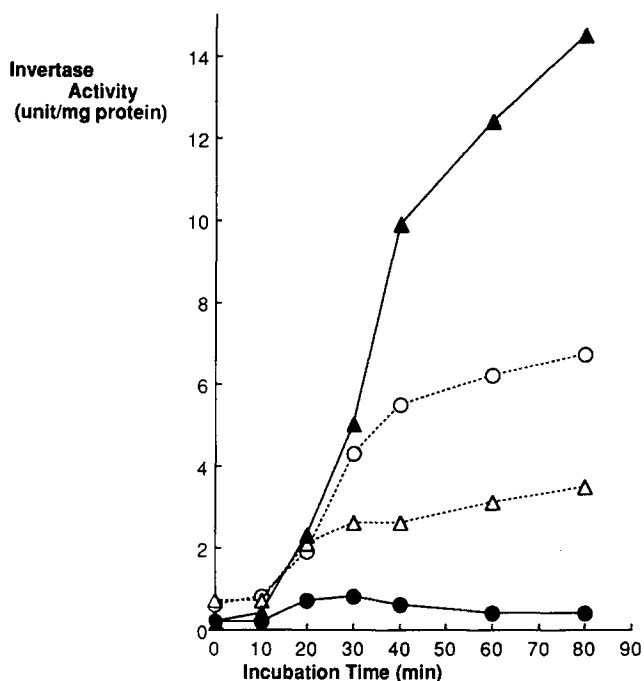


Figure 1. Cessation of invertase secretion by expression of the *usol* Ts-defect. Strain USO1-3 (*usol*, *SUC2*) and X2180-1A (*USO1*, *SUC2*) were cultured in YEP7D medium at 25°C. The cells were shifted to YEP2S medium and incubated at 37°C. At the indicated times after temperature shift, aliquots were removed and intracellular and extracellular invertase was assayed. Open symbols indicate extracellular invertase pools; closed symbols indicate intracellular pools. (circles) Wild type cells; (triangles) *usol* cells.

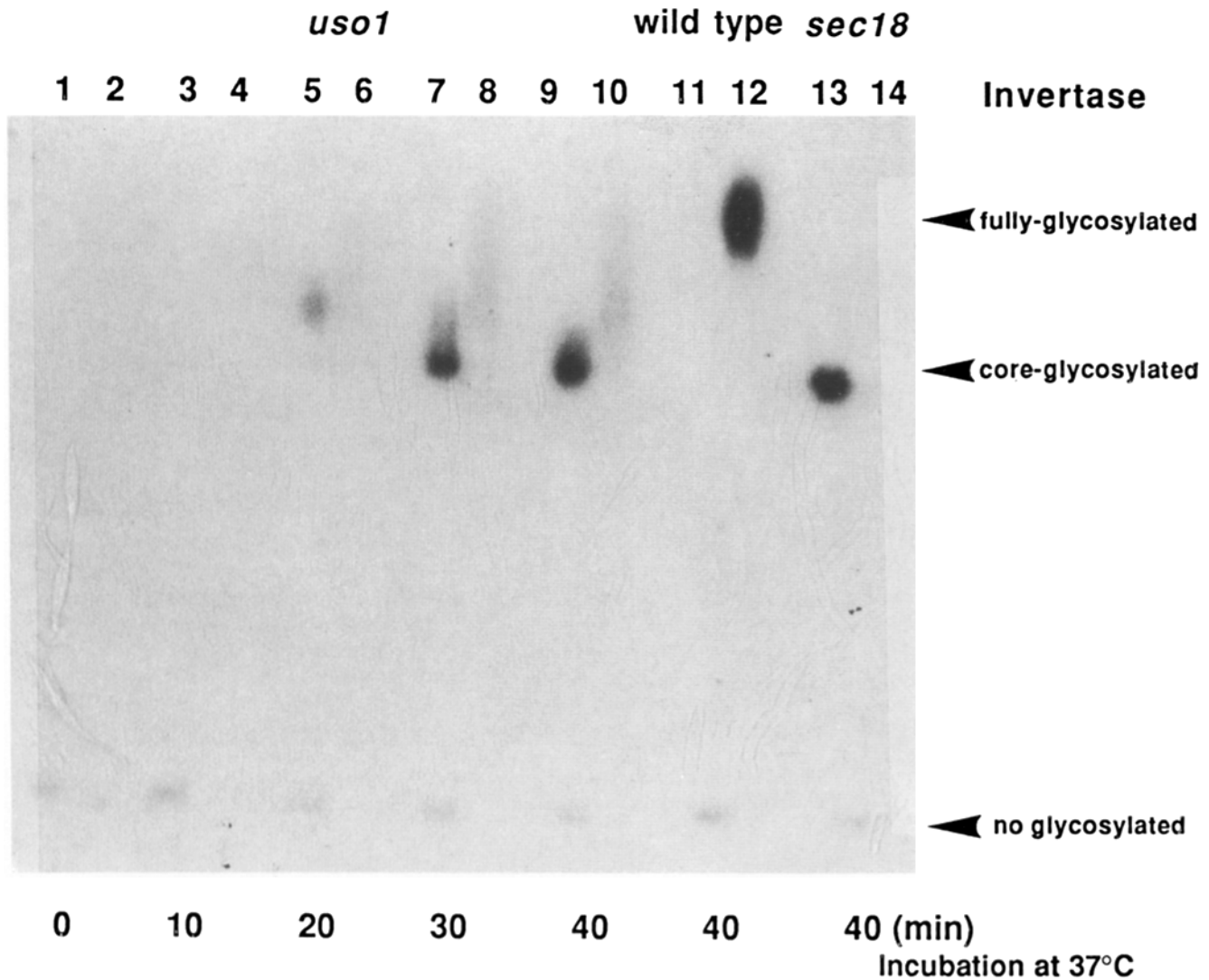


Figure 2. Accumulation of core-glycosylated invertase in the *usol* cells. *usol* (lanes 1-10), wild type (lanes 11 and 12), and *sec18* (lanes 13 and 14) cells were grown in YEP7D media at 25°C overnight to 1.0×10^7 cells/ml. Invertase was induced by transferring the cells to YEP2S medium; at the same time incubation temperature was shifted to 37°C. At the indicated times, cells were collected and spheroplasted to fractionate periplasmic and intracellular pools. The fractions were heated at 50°C for 10 min and subjected to electrophoresis on native 6.75% polyacrylamide gel that was subsequently stained for invertase activity. Intracellular invertase is in lanes 1, 3, 5, 7, 9, 11, and 13; extracellular invertase is in lanes 2, 4, 6, 8, 10, 12, and 14. Nonglycosylated, core-glycosylated, and fully glycosylated invertase correspond to 59, 80, and 110-130 kD, respectively, in molecular mass.

plasts, which were spun to separate the contents of the cells from the fraction released into the periplasm. The activities of invertase in the periplasmic and the intracellular fractions are shown in Fig. 1. In the wild type cells, intracellular invertase activity did not increase from the constitutive level. Most of the newly synthesized invertase was transported to the cell surface rapidly. On the contrary, the *usol* cells transported a small amount of invertase to the cell surface during the first 20 min at the restrictive temperature, but began to accumulate active invertase intracellularly on further incubation at 37°C. To examine the glycosylation pattern of invertase accumulated in the *usol* cells, intracellular and periplasmic fractions were subjected to electrophoresis on nondenaturing polyacrylamide gels in which invertase activity was detected in situ (Fig. 2) (Meyer and Matile, 1974).

The intracellular fractions always showed the nonglycosylated form of invertase which is constitutively expressed and retained in the cytoplasm. The wild type cells secreted fully glycosylated invertase into the periplasm (lanes 11 and 12), but the *sec18* (ER accumulation type of secretion mutant) cells accumulated core-glycosylated invertase intracellularly during 40 min incubation at 37°C in the depression medium (lanes 13 and 14). The *usol* cells secreted invertase into the periplasm that had a lower extent of glycosylation than the wild type, and accumulated even less glycosylated invertase in the intracellular fraction during the first 20 min incubation at 37°C (lanes 5 and 6). On further incubation, the core-glycosylated form of invertase was accumulated in the intracellular fraction (lanes 7-10). Therefore, the secretory protein in this mutant seems to be accessible to the core-glycosyla-

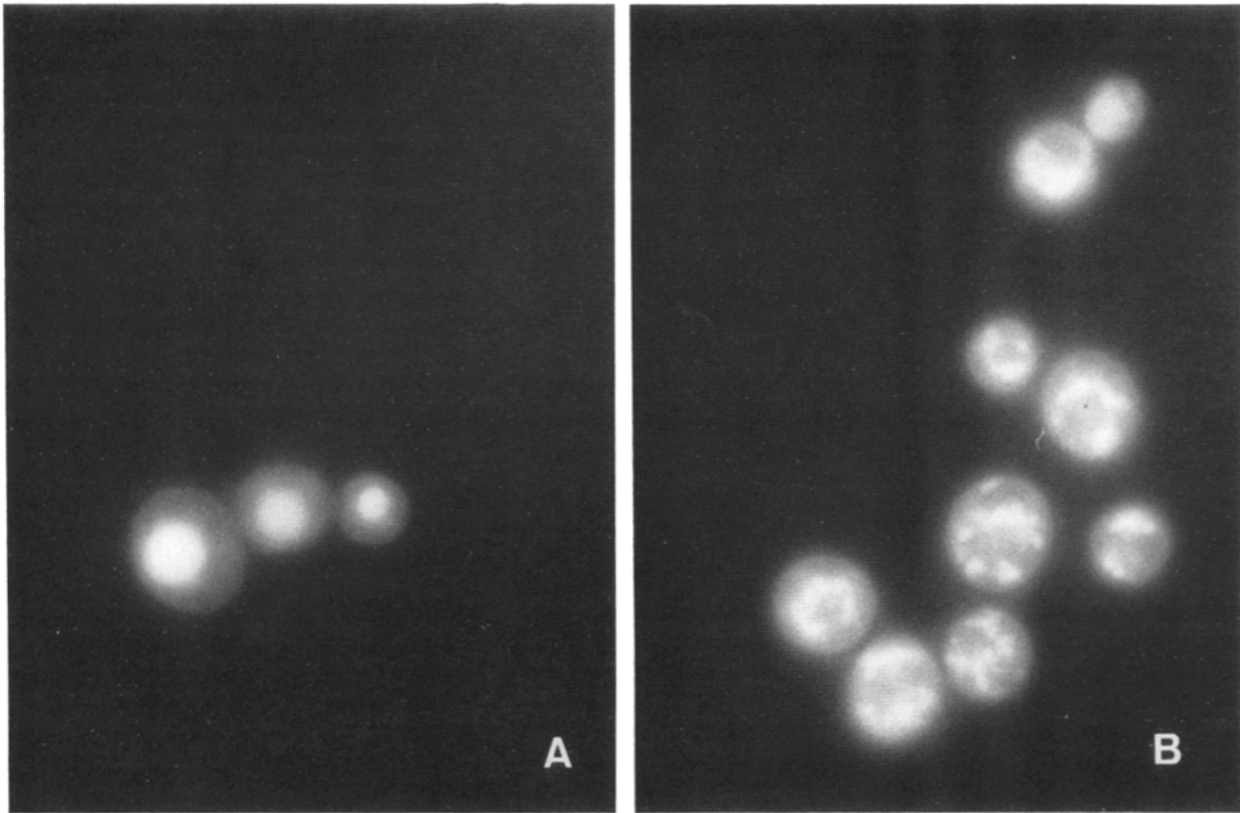


Figure 3. Fluorescent micrograph of chloroquine-stained wild type (A) and *usol* (B) cells. Cells were grown at 25°C and preincubated at 37°C for 1 h. Chloroquine was taken up during a subsequent incubation at 37°C for 30 min and accumulated in the low pH organelle: vacuole.

tion machinery of the ER, but not to the outer-chain addition machinery of the Golgi apparatus. Perhaps it takes 20–30 min for the *usol* mutant cells to block the transport of the protein from the ER to the Golgi.

These observations indicate that the *usol* cells can translocate invertase into the ER and core-glycosylate it, but transport of the protein from the ER to the later secretory steps is blocked and only the core-glycosylated invertase is accumulated in the cell.

Vacuole Fragmentation in the usol Cells

The *usol* cells arrest increase of cell number 30 min after transfer to the restrictive temperature. Observation by phase-contrast microscopy showed that the *usol* mutant strain did not accumulate cells arrested at a specific stage of the cell cycle during incubation at 37°C, indicating that the *usol* mutation has no direct effect on the regulation of the cell cycle control. Under the phase-contrast microscope, large vacuoles were clearly observed in the wild type cells but not in the *usol* cells. To observe the vacuole morphology, wild type and *usol* cells were preincubated at 37°C for 1 h and incubated with 20 mM chloroquine at 37°C for 30 min. Chloroquine is a fluorescent reagent that is taken up by living cells and accumulates in low pH organelles, such as vacuoles (Lenz and Holzer, 1980). Fluorescent micrographs showed that wild type cells have one large vacuole (Fig. 3 A), but *usol* cells have a large number of vesicles arranged in a circle in

the cytoplasm (Fig. 3 B). *secl2* and *secl8* cells showed similar morphology of vacuoles with the *usol* cells (data not shown).

usol Cells Accumulate the Expanded ER and Nuclear Envelope

Previously reported *sec* mutants have been shown to accumulate secretory organelles at the restrictive temperature. To observe the intracellular membrane structure of the *usol* mutant strain, we applied the rapid freeze-substitution method to minimize the artificial changes that could occur during fixation by conventional methods (Kanbe and Tanaka, 1989). In the *usol* cells incubated at 25°C, completely round nucleus and thin cisternae of the ER and the nuclear envelope were observed (Fig. 4 A). Wild type cells incubated at 37°C showed the same feature (data not shown). The lumen of the ER and the nuclear envelope ranged 15–20 nm in width (Fig. 4 B, *face to face triangles*). When *usol* cells were incubated at 37°C for 2 h, several distinctive features were observed. First, the ER developed remarkably and formed a complex structure like a network (Fig. 4 C). The accumulated ER shown in Fig. 4 D had contacts with the plasma membrane, and the lumen of the ER and the nuclear envelope were expanded to 45–50 nm in width (Fig. 4 D, *triangles*). In addition, the nucleus with unusual morphology and several fragmented vacuoles were often observed in the *usol* cells (Fig. 4, C, D, E, and I).

By applying the freeze-substitution fixation method, we could detect the microtubules more clearly than with conventional methods. Yeast cells contain typical eukaryotic microtubules and they apparently emanate from the face of the SPB, a microtubule-organizing center embedded in the nuclear envelope (Byers, 1981). The *usol* cells incubated at 37°C for 2 h often showed two bundles of microtubules (Fig. 4 E), one being normally orientated along the long axis of the cell and associated with the SPB on the nuclear envelope, and the other not binding to SPB. These abnormal microtubules were tightly bundled to each other to form a crystalloid structure in the nucleus (Fig. 4, C and F). Fig. 4 G shows a cross section of the closely bundled microtubules. Compared to the microtubules of wild type cells (Fig. 4 H), the bundle of microtubules were packed more tightly in *usol*. Remarkable bundles of microfilament were also found in many *usol* cells. Fig. 4 I shows that bundles of filaments, 50–100 nm in diameter, traverse across the cytoplasm. By the fluorescent microscopy stained with rhodamin-labeled phalloidin (Adams and Pringle, 1984), wild type cells showed actin spots and thin fibers orientated along the long axes, but *usol* cells often showed abnormally bright actin fibers (data not shown). These observations suggest that the *usol* mutation induces not only accumulation of intracellular membrane organelles, but also formation of abnormal morphology of cytoskeleton-related components: microtubules and actin filaments.

Cloning of *USO1* Gene

To investigate the role of the *USO1* gene in the process of protein transport from the ER to the Golgi, we planned to clone the wild type *USO1* gene. A yeast genomic DNA clone that complements the temperature-sensitive phenotype of *usol* was isolated from a DNA library constructed on the single copy vector YCpG11, a shuttle plasmid carrying a yeast centromere (*CEN4*) and the *TRP1-ARS1* gene. Strain USO1-10 (a, *usol-1*, *ura3*, *leu2*, *trp1*) was transformed with the library DNA and transformants were selected by Trp⁺ and Ts⁺ growth. From ~10⁴ transformants, 12 candidates were recovered from the same group of DNA library. A plasmid pHN155 was recovered from one of the candidates, and USO1-10 strain was retransformed. All the transformants selected by Trp⁺ acquired temperature resistance. Restriction enzyme mapping showed that the plasmid contains a 9.5-kb yeast DNA insert. The full length of the insert was subcloned into a multicopy vector and introduced in the strain USO1-10. All Trp⁺ transformants showed temperature resistance and grew at a normal rate, indicating that multiple dosage of this fragment did not have any poisonous effect to the host cells. By a combination of deletion mapping and subcloning analysis, the complementation activity of the pHN155 insert was localized to a 4.5-kb SmaI–XhoI restriction fragment. The essential region for the complementing activity was shortened to a 3.3-kb fragment by further subcloning analysis of successive deletions (Fig. 5, fragment A).

To confirm that our clone carried the authentic *USO1* and not a suppressor, we first constructed the integrating plasmid pHN163 by inserting the cloned 9.5-kb fragment of pHN155 into the YIp5 integrating vector containing the *URA3* gene. This plasmid was cut at the unique SacI site within the cloned DNA to direct integration into the homologous chromosomal

locus and introduced into the wild type strain DBY747 (a, *ura3*, *USO1*). One of the Ura⁺ transformants, DBY747-U, was mated with the mutant strain USO1-24 (α , *ura3*, *usol-1*), and the resulting diploid strain DBY747-U/USO1-24 (a/a, *ura3/ura3*, *USO1::URA3/usol-1*) was sporulated. In an analysis of 35 randomly separated spores, 15 colonies showed Ts⁺ Ura⁺ and 18 colonies showed Ts⁻ Ura⁻. This indicates that pHN163 (*USO1::URA3*) integrated at or near the *USO1* locus in the chromosome (Orr-Weaver et al., 1981).

Secondly, we constructed the *USO1* gene disruption plasmid by replacing 2.5-kb EcoRV–XhoI fragment in the *USO1* gene with 1.8-kb Sall–SmaI fragment containing the yeast *URA3* gene (Fig. 5). This disrupted allele was introduced into the heterozygous diploid strain DBY747/USO1-24 (a/a, *ura3/ura3*, *USO1/usol-1*) by one-step gene replacement (Rothstein, 1983). Among the resulting 7 Ura⁺ transformants, four showed Ts⁻ phenotype, consistent with disruption of *USO1* gene (a/a, *ura3/ura3*, Δ *usol::URA3/usol-1*). These results indicate that the cloned yeast DNA is the wild type *USO1* gene. To determine whether the *USO1* gene performs an essential function in yeast, we assessed the fate of haploid progenies of this diploid which was heterozygous for *USO1*. The disrupted heterozygous strain (a/a, *ura3/ura3*, *USO1/Δusol::URA3*) was sporulated and tetrads were dissected. Among 15 asci analyzed, 10 gave rise to 2 viable spores and 5 produced only one viable spore. All of the viable spores were Ura⁻. This result indicates that the *USO1* gene is essential for yeast cell growth.

DNA Sequence of *USO1*

The nucleotide sequence of each strand of the 3.4-kb fragment that contains *usol* complementing activity and its flanking DNA region was determined by the didexonucleotide chain-termination method (Fig. 6). There is only one long open reading frame (ORF) of 5,370 nucleotides (1,790 codons) that is not interrupted by introns in this DNA region. To our surprise, the reading frame was longer than the minimal fragment containing *usol* complementing activity. This indicates that the truncated *USO1* gene is sufficient to support growth of the *usol-1* mutant even when one-third of its COOH-terminal part is deleted. To confirm the size and orientation of the *USO1* ORF, we constructed a gene fusion of the *E. coli* *phoA-lacZ* fusion gene promoter and NH₂-terminus 400 codons to the ClaI site (amino acids 151–152) of the *USO1* ORF. The resultant fusion gene was expressed in *E. coli* by phosphate limitation: an expected size of fusion protein was shown (data not shown). A potential transcription initiation signal (TATA box) is noted at 22 nucleotides upstream of the putative initiator AUG (Fig. 6).

A highly hydrophilic polypeptide with a molecular mass of 206 kD (containing 35% charged amino acids) is predicted from the ORF. Hydropathic analysis of the putative Usolp was performed according to the predictions of Kyte and Doolittle (1982) (Fig. 7). No obvious structure resembling a classical signal peptide was discovered at the NH₂-terminus and no hydrophobic amino acid stretch long enough to span the membrane was detected in the entire sequence. This suggests that Usolp itself is neither a passenger of the secretory pathway nor an integral protein of secretory organelle membranes. On the other hand, the region between residues 465 and 485 exhibited hyper-hydrophilicity due to

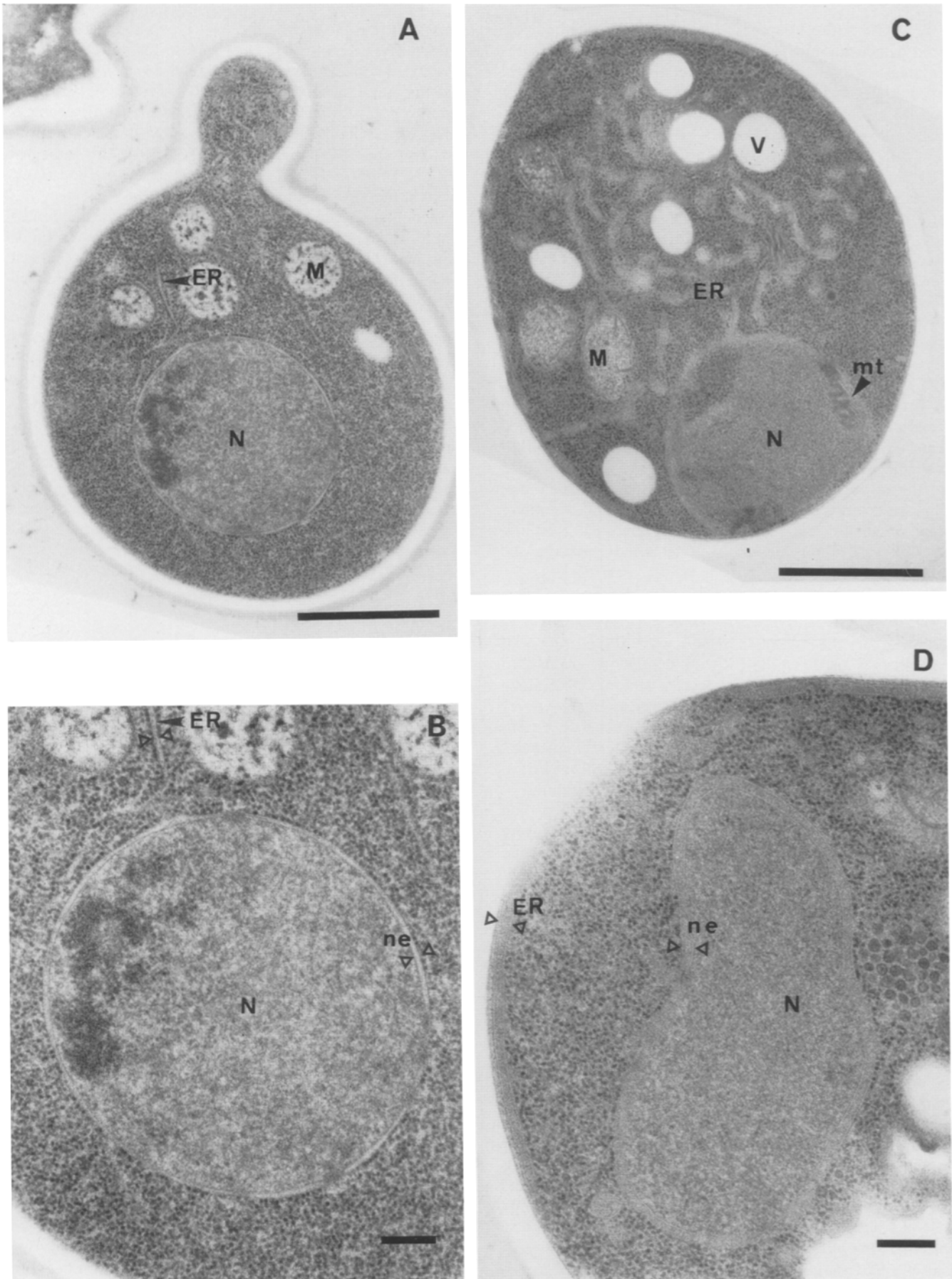
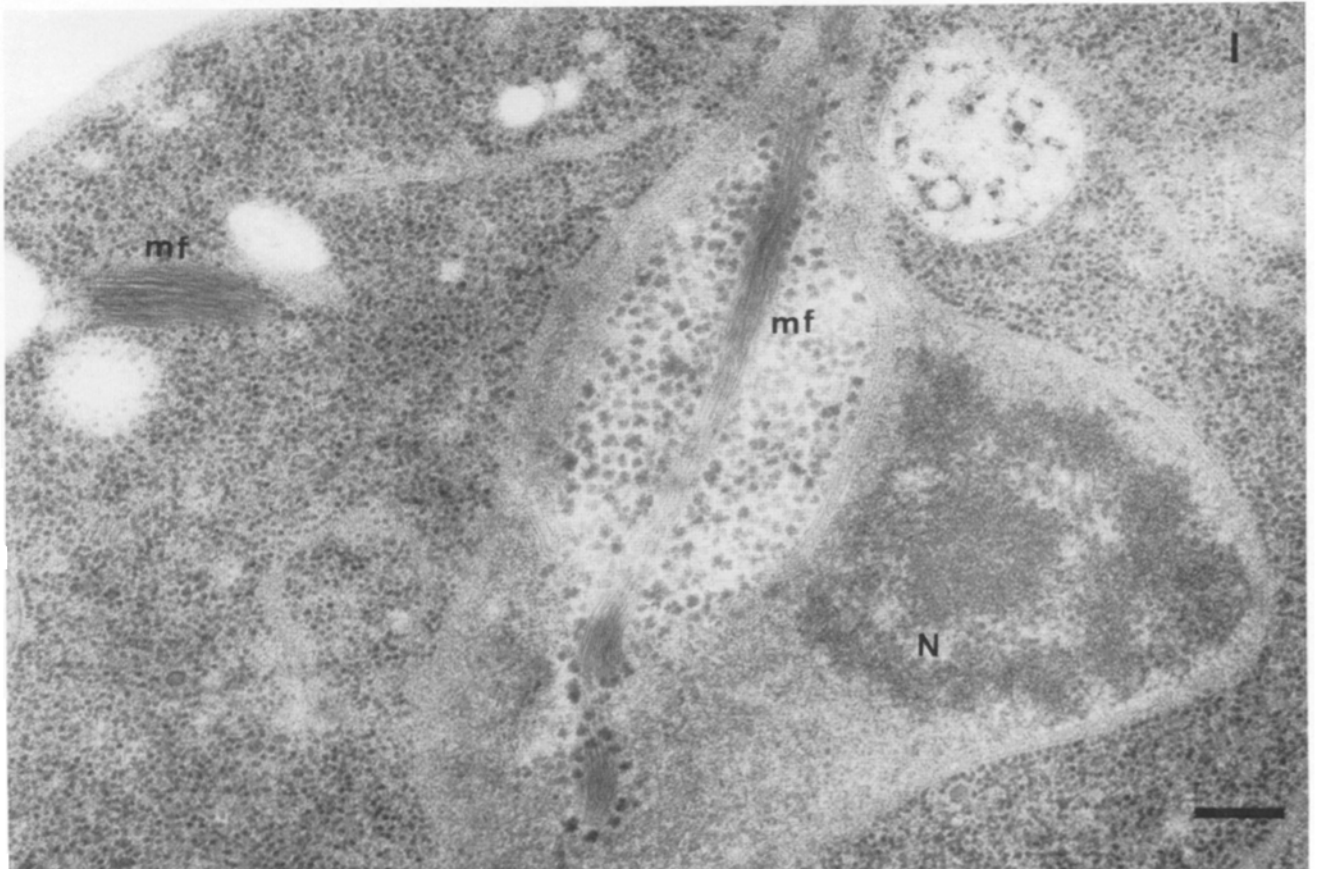
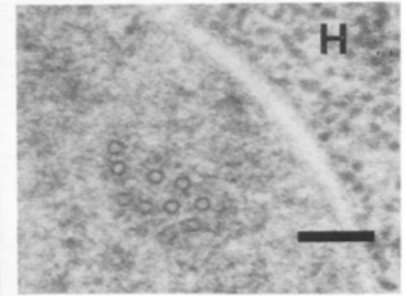
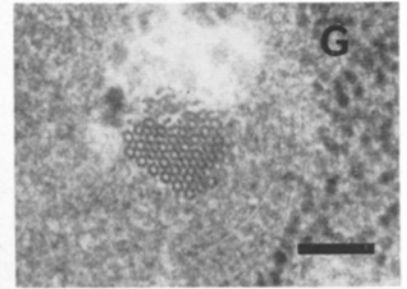
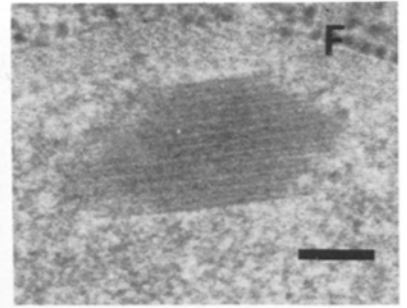
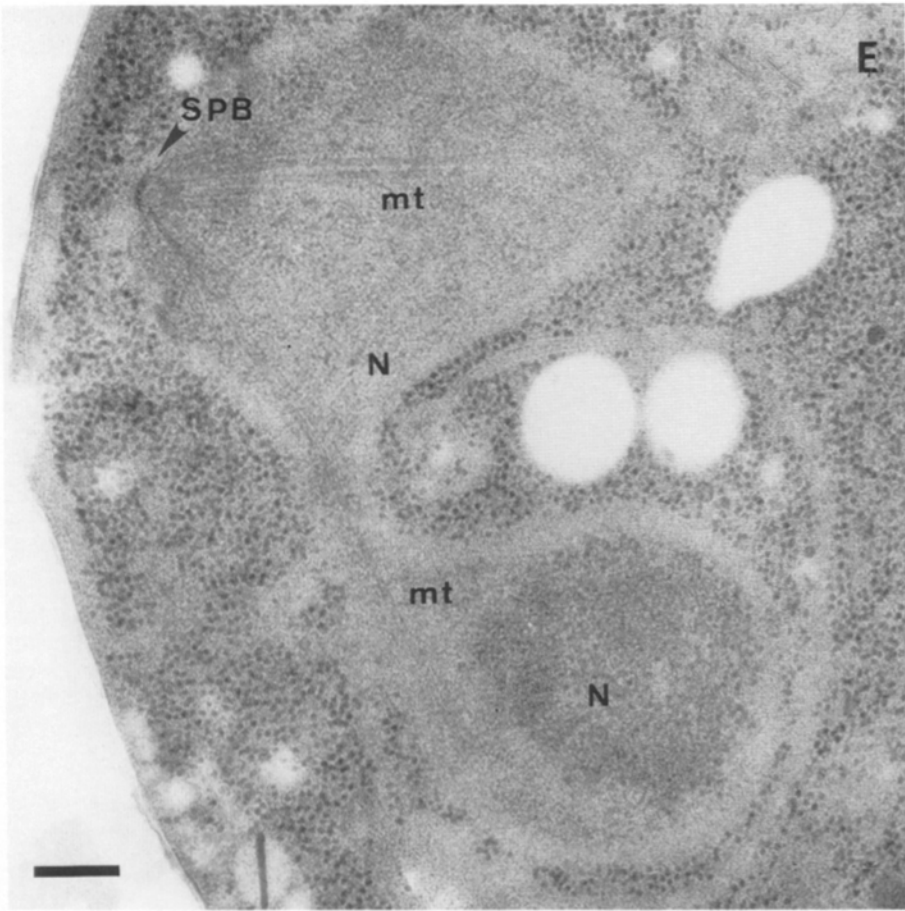


Figure 4. Electron micrographs of wild type and *usol* cells fixed by the freeze-substitution method. *usol* cells incubated at 25°C (A and B) and at 37°C for 2 h (C, D, E, F, G, I). Wild type cells incubated at 37°C for 2 h (H). (N) nucleus; (V) vacuole; (M) mitochondria; (ne) nuclear envelop; (SPB) spindle pole body; (mt) microtubules; (mf) microfilament. Bars: (A and C) 1 μm ; (B, D, E, and I) 0.2 μm ; (F, G, and H) 0.1 μm .



703 AGTTATTTAATGAAAACATATACAGGATCAGAACTGCATTGTCTCACGCCCTGACGAAG
 L F N E N I Y R I R T A L S H D P D E E 1463 AACGAGACCTGGAGACTTGAAGAACAACCTCCGGCTGCACAAGAATCCAAAGCTAAAG
 R D L E S L K E Q L R A A Q E S K A K V
 723 AGCCCAATCAGTAAATATCTTTGAAAGAACTGAAAATACAGAGGCAATGTACAAAAT
 P I S K I S F E V E K L Q R E C Q C T K L 1483 TTGAAGAAGGCTGAAAAAATCTGAGGAAGAACTTCAAAAAGAGAAGCAGAGCTTGAAA
 E E G L K K L E E E S S K E K A E L E K
 743 TGAAGGGTGAATACTTCTTTCGAAACAGAAACGAAAGCACCATGAGAACCTCACCG
 K G E I T S L Q T E T E S T H E N L T E 1503 AGTCTAAAGAAATGATGAAAAAGCTAGAGAGTACGATTGAGAGCAATGAAACGGAGTTGA
 S K E M M K K L E S T I E S N E T E L K
 763 AAAAATGATTGCGTTGACTAATGAACACAAAGAGTTGGATGAGAAATACAAAATTTTGA
 K L I A L I N E H K E L D E K Y Q I L N EcoRI
 1523 AATCCTCAATGGAAACCATAAGGAAATCAGATGAAAAATGAGAACGTCGAAAAAATG
 S S M E T I R K S D E K L E Q S K K S A
 EcoRV
 783 ATTCTCACATTCTCGTTGAAAGAAAATTTCCATTTTGGAACTGAATTTGAAGAACG
 S S H S S L K E N F S I L E T E L K N V 1543 CTGAGGAGGATATTAATACTTGAACATGAGAGTCAAGATTTGATATCCGGAATCAACG
 E E D I K N L Q H E K S D L I S R I N E
 803 TCAGAGATTCCTGGATGAATGACGCAACTGAGAGATGTACTGGAACTAAGGACAAAG
 R D S L D E M T Q L R D V L E T K D K E 1563 AATCTGAAAGGATATTGAAAGCTGAAAGTAAACTGAGGATCGAAGCAAAAATCTGGCT
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 823 AAAATCAAACTTTTACTGGATACAAAAGCAACTCCACAACAAGAAGACTCTATCA
 N Q T A L R Y K E A A T T C H A C K Q E D S I K 1583 CTGAACTGAAACCGTAAAAACAAGAGCTAAATAACGCCACAAGAAAAATAAGGATCAACG
 E L E T V K Q E L N N A Q E K I R I N A
 843 AAACCTTAGAAAAAGGACTTGAACATTTTGTCTCAAAGAAAAGGCGAGAAGATGGTA
 T L E K G L E T I L S Q K K A E D G I 1603 CAGAAGAAAATACAGTTTGAATCTAAATAGAAATATAGAAGTGAACGTAACAAAGACA
 E E N T V L K S K L E D I E R E L K D K
 863 TAAACAAAATGGGTAAGATTTATTCGCTCTGAGTAGAGAGATGCAAGCAGTTGAGGAGA
 N K M G K L F A L S R E M Q A V E E N 1623 AGCAAGCCGAAATAAAAAGTAAATCAAGAAGAAAAGAAATTAACCTACCTCGCCCTCAAG
 T A G E I K S N Q E K E L L T S R L K E
 883 ATTGTAAAATTTACAGAAGGAAAAGATAAAGCAATGTCAACCATCAAGAGAGACTA
 C K N L Q K E K D K S N V N H Q K E T K 1643 AATTAAGCAGGAATTAGATAGCACCCACAGAAGGCACAAAATCCGAGGAAGAACGTA
 L E Q E L D S T Q Q K A Q K S E E E R R
 903 AATCACTAAAAGAAATATTCGCGCAAAAATTTACTGAAATAAAGCTATCAATGAAATC
 S L K E D I A A K I T E I K A I N E N L 1663 GGGCCGAACTCAGGAAGTTCAAGTCGAAAAGCTCAACTTGTGAAAAGCCATGTTGC
 A E V R K F Q V E K S Q L D E K A M L L
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 E E M K I Q C N N L S K E K E H I S K E 1683 TTGAACTAAATCAACAGTTTGGTGAATAAAGAGCAAGCATGGAAAAGGATGAGATA
 E L E T V K Q E L N N A Q E K I R I N A
 943 AACTTGTGAGTACAATCCCGCTTTCAGAGTCAAGCAATCTAGTAGCGAAACTAACAG
 L V E Y K S R F Q S H D N L V A K L T E 1703 CTGTTAAAAGACAACCGATTCTCAGAGACAAGAAATGAAAAGTTGGCTAAAGAACTGG
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 K L K S L K N L S K M Q A E N S E I D 1723 ATAACCTGAAGGCAGAGAACTTAAGTTGAAGGAAGCTAACGAAGATCGTTCCGAAATG
 N L K A E N S K L K E A N E D R S E I D
 983 TAAAAGCTGTAGAAGAGTCAAAAACGAAAGCAGCATAAATGCTAATTTGCAAAAATA
 K A V E E S K N E S S I Q L S N L Q N K 1743 ATGACTTGTAGTCTTGGTTACTGACCTAGATGAGAAAAACGCCAAATATCGCTCAAAGC
 D L M L L V T D L D E K N A K Y R S K L
 1003 AAATGATTTCTATGTCACAGGAAAAGAAAATTTTCAATAGAAAAGGCGAGTATAGAAA
 I D S M S L Q E K E N F Q I E R G S I E K 1763 TGAAGGATTTGGGCGTTGAAATAGTTCCGATGAAGGAGGATGATGAAGAAGATGATGAAG
 K D L G V E I S S D E E D D E E D D E E
 1023 AAAATATCGAACTAAAACCACTCTCTGACTTGAACAACGAAAGGAGGAAATTA
 N I E Q L K K T I S D L E Q T K E E I I 1783 AAGACGAGGAAGAGGCAAGTGGCATGTAGAGTTAAAATATGTGCATAAACAATCGC
D E E E G Q V A *
 1043 TCTCAAAATCCGATTTCTCAAAGATGAATATGAGTCCCAGATTAGTCTCTTGAAGAAA
 S K S D S S K D E Y E S Q I * S L L K E K
 1063 AATTAGAAACGGCGACAACAGCAACAGTGAACAACTGAAATTAATCTCAGAATGACTA
 L E T A T T A N D E N V N K I S E L T K
 1083 AAACCTAGGAAAGAGCTGGAAGCCGAGTTAGCAGCTTATAAAAATCTCAAAAATGAACTG
 T R E E L E A E L A A Y K N L K N E L E
 HindIII
 1103 AAACAAAATAGAACTTCAAGCAAGCTTGAAGAACTTAAAGAAAACGAGGAGCAT
 T K L E T S E K A L L G K E V K E N E E H
 XhoI
 1123 TAAAAGAGAGAAAATCAACTCGAGAGGAGGCACTGAAACCAACAGCAACTCAACA
 K E E K I Q L E K E A T E T K Q Q L N S
 1143 GCCTACGTGCCAATTTAGAATCATTAGAAAAGAGCATGAAGATTTAGCAGCTCAGTTGA
 L R A N L E S L E K E H E D L A A Q L K
 1163 AGAAGTACGAGGCAAAATGCCAACAGGAAAGCAATATAACGAGGAATATCTCAAT
 K Y E E Q I A N K E R Q Y N E E I S Q L
 1183 TAAATGATGAGATTACTTCCACTCAACAGAAAATGAATCAATTAAGAAAAAATGAGC
 N D E I T S T Q Q E N E S I K K K N D E
 1203 AACTGGAGGCGAAGTTAAAGCAATGAAGAGCACTTCAAGGAAACAAATTTGAAA
 L E G E V K A M K S T S E E Q S N L K K
 1223 AGTCAAGATTTGATGCCTTAAATTTGCAAAATTAAGAGCTGAAAAGAAAGAACGAAACGA
 S E I D A L N L Q I K E L K K K N E T N
 1243 ACGAAGCCAGTTTATGGAGTCAATCAAAAGTGAAGATCAGAAACGGTAAAAATTAAG
 E A S L L E S I K S V E S E T V K I K E
 1263 AATTACAAGACGAATGTAACCTTAAAAGAAAAGAAAGTTAGTGAATTTGAAGCAAACTCA
 L Q D E C N F K E K E V S E L E D K L K
 1283 AGGCCTCAGAAGATAAAGACTCTAAATATTTGGAGCTACAGAGGAATCGGAGAAGATAA
 A S E D K N S K Y L E L Q K E S E K I K
 1303 AAGAAGACTAGATGCTAAAACGACTGAACTAAAAATCAATTTGAAAAGATCACTAATT
 E E L D A K T T E L K I Q L E K I T N L
 XbaI
 1323 TATCTAAGCCAAAGGAGAAATCAGAACTGAGTTGCTTGAATTGAAGAAGACATCTCTG
 S K A K E K S E S E L S R L K K T S S E
 1343 AGGAAAGAAAATGCAGAGGAGCAATTTGAAAATTTGAAAATGAAATCAAAATCAAAA
 E R K N A E E Q L E K L K N E I Q I K N
 1363 ATCAGGCCCTCGAAAAGGAAAGAAAGCTACTTAATGAAGGATCTTCAACAAATACACAAG
 Q A F E K E R K L L N E G S S T I T Q E
 1383 AATATTCTGAAAAGATCAACTTTGGAAGATGAATTAATAGGCTACAATAAGAAAAGC
 Y S E K I N T L E D E L I R L Q N E N E
 1403 AATTAAGCCAAAGAGATCGATAATACAGGAGTGAACAGAGGATGAGTTAGTTAAGCA
 L K A K E I D N T R S E L E K V S L S N
 HindIII
 1423 ATGATGAGCTTTTAGAAGGAAACAAAACACAATAAAAGCTTACAAGATGAAATCTTT
 A D E L E A E K Q N T I K S L Q D E I L S
 HindIII
 1443 CATATAAGCAAAAATACTAGAAATGACGAAAGCTTCTCAATGAGGCGAGATAATA
 Y A K D K I T R N D E K L L S I E R D N K

Figure 6. Nucleotide sequence of *USO1*. The DNA sequence of the *USO1* coding strand and the predicted primary sequence of its product are shown. Potential promoter TATA box is marked with a box; hyper hydrophilic amino acid stretch is marked with underline; cluster of acidic amino acids is marked with double underlines. Minimal *uso1* complementing fragment, Fragment A in Fig. 5, is between ** at the 45-bp upstream of the first ATG and ** at the 1057th amino acid. These sequence data are available from EMBL/GenBank/DBJ under accession number X54378.

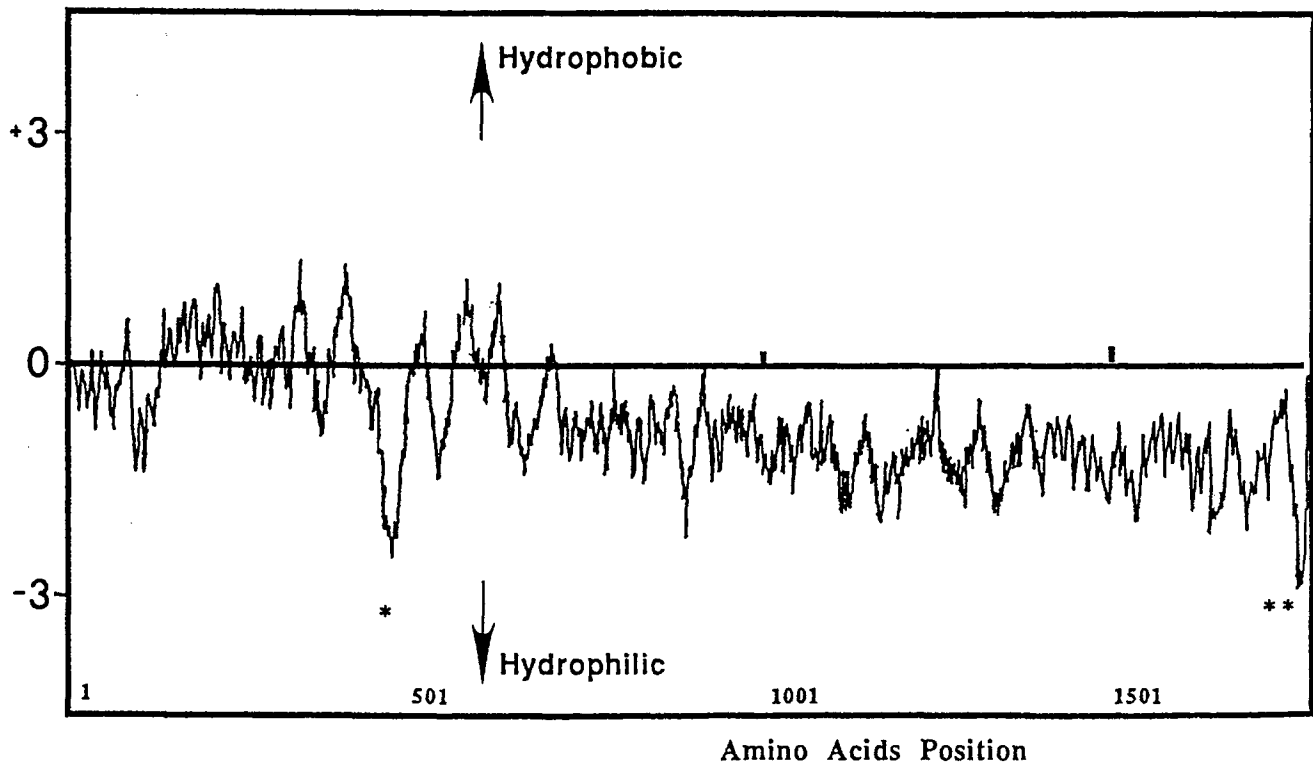


Figure 7. Hydropathy analysis of Usolp. The inferred Usolp amino acid sequence was used to generate a hydropathy profile by the method of Kyte and Doolittle (1982). A scanning window of 20 residues was used. Amino acid residues are numbered along the horizontal axis while net hydrophobicity values are indicated on the vertical axis. * indicates the hyper hydrophilic amino acid stretch and ** indicates the cluster of acidic amino acids shown in Fig. 6.

peat units are present in the rod regions of these proteins and hydrophobic amino acids located at the first and the fourth positions in the units (McLachlan and Karn, 1983). Fig. 9 shows the 7-amino acid repeat units of Usolp (Yang et al., 1989). The COOH-terminal 1,100 amino acids of Usolp are lined up in every seven amino acids (Fig. 9 A), and the number of hydrophobic amino acids at each position in the units (a, b, c, d, e, f, and g) is shown in Fig. 9 B. The most hydrophobic amino acid, leucine, is most markedly biased at the positions a and d. In addition, this Usolp COOH-terminal region contains no proline and only 10 glycine residues, both of which are strong α -helix breakers. In fact, it is calculated that $\sim 90\%$ of this region may form the α -helical secondary structure (data not shown) (Chou and Fasman, 1978). These results strongly suggest that COOH-terminal 1,100 amino acids of Usolp form an α -helical rod domain.

Discussion

A sexual agglutination selection has been used to isolate a new temperature-sensitive mutation *usol* which blocks early in the yeast secretory pathway. This selection method is expected to concentrate the mutant cells which have a defect in the process of pheromone response, protein synthesis, or protein secretion. The mutant *usol* cells secrete invertase for the initial 20 min after shift up to the restrictive temperature, and then begin to accumulate it in the cell (Fig. 1). This indicates that the block of secretion is invoked more slowly than the induction and secretion of invertase. Similar to most of

the previously reported *sec* mutants, *usol* cells produce more than two times as much level of invertase as the wild type when incubated at the restrictive temperature (Novick et al., 1980). Accumulated invertase in the *usol* cells is in the core-glycosylated form after 30 min of incubation at 37°C (Fig. 2, lanes 7 and 9), but at 20 min incubation slightly more glycosylated invertase is found in the cell (Fig. 2, lane 5). It seems that slow down of late Golgi secretory processes occurs earlier than transport block from the ER to the Golgi step in the *usol* cells at the restrictive temperature. The mutant *usol* accumulates the ER, and the lumen of the ER and the nuclear envelope are expanded by two to three times in width by 2 h incubation at the restrictive temperature. The volume of ER and nuclear envelope increases more than 10 times. Compared to the core-glycosylated form of invertase in the *sec18* cells, the *usol* cells accumulate slightly more diffuse and higher molecular weight forms of invertase (Fig. 2, lanes 9 and 13). It may be the reflection of the difference of genetic background between the strains. However, we cannot deny the possibility that the *usol* cells accumulate the invertase containing outer chain carbohydrate. Possibly the newly synthesized secretory proteins are accumulated in the subcompartment between the ER and early Golgi complex, or they could be once transported to the Golgi stacks and flowed back to the ER since the later secretory pathway is blocked in the *usol* cells. The *sec18* cells secreted the accumulated precursor proteins within 1 h when the cells were shifted down to the permissive temperature, but the secretion ability of *usol* cells was restored slowly and incompletely

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Usolp 652'  TLGKDNVYASRIKQPKKDSYFSKVDNMEDSILTPELDETLGPKVYFSTYFIQLFNENIYRI
Myosin 781'  VYKQAREHTVAARI IQQNLRLAYIDFKSWPWWKLFKSKARP LLKRRNFEEKEIKEREIELEL
712'  RTALSHDPDEEPI SKISFEVEE-KLQRQCTKLKGEITSLQTETESTHENLTKELTALTNE
841'  KSNLTDSTTQKDKLEKSLKDTESNVLDLQRQLKAEKETLKAMYDS-KDALEAQKRELEIR
771'  HKELDEKYQILNSHSSLNKFNFSILETELKQVRESLDEMTQLRDVLETKDKENQATALLEY
900'  VEDMESELDEKKLALENLQNKRSVEEKVRDLEELQEEQKLRNTELEKLLKQYEEBLEEM
831'  KSTIHKQEDSIRKLEKLETLISLQKKAEDGINKMKDILFALSREMQAVEENCKNLQKEK
960'  KRWNQDQSDTISRLEKIKDELQKVEBELTESFSESKDKGVLEKTRVRLQSELDL-TVR
891'  DKSNNVHQKETS LKEDIAAKITEIKAINENLEEMKIQCNNSKEKEHISKELVE-YKSR
1019'  LDSETKDKSELRLQKKELEELKQVQEAALAAETAALKLAQAANKLQGEYTELNEKFNSE
950'  FQSHDNVLAKLTKELKS-LANNYKDMQAEENSLIKAVEESKNESIQLSNLQNKIDSMQ
1079'  VTARSN-VEKSKTTLESQLVAVNNEDELDEKKN-RDALEKKAALDAMLEMKQDLESTGG
1009'  EKENFQIERGSEIKNIEQLKKTISDLQTKKEEISKSDSSKDEYESQISLLKEKLETATT
1137'  EKKSLYDLVKQESDMEALRNQISEL---QSTIAKLEKIKSTLEGEVARLQGELEAEQL
1069'  ANDENVNIKISELTKREELEAELAAAYKNLKNLETKLETSEKALKEVKEENEHLKKEKIQ
1193'  AKSNVEKQKKVELDLEDKSAQLAETAARKQALDKSKKSEQELSEVQTQLSEANNKNVN
1129'  LEKEATEKQQLNSLRANLESELEKHEDLAAQLKQYEEQIANKERQYNEEISQLNDEITS
1253'  SDSTNKHLETSFNNLKLLEAEQAKQALEKRLGLSESELKHVNEQLLEEKQKESNEKR
1189'  TQQENESIKKNDLEGEVYKAMKSTSEEQSNLKSSEIDALNLQIKELKKKNETNEASL--
1313'  KVDLEKEVELSKDQIEEVEASKAVTEAKNK-KESELDEIKRQYADVVSRRDKSVEQLKT
1247'  LESIK-SVESETVKIK-ELQDECNEKEVSELEDKKAEDKNSYLELQKESSEIKKEE
1372'  LQAKNEELRNATAEAEQGLDRAERSKKAEFLEEAVERNLEETAKKVKAEKAMKKAETO
1305'  LDKATTELKIQLEKINTLSKAKESELSRLK--TSSEER-----KNAEEQLEKL
1432'  YRSTKSELDDAKNVSEQYVQIKRLNEELSELRSVLEEADERCNSAIKAKKTAESALESL
1355'  KNEIQKNAQFEKERKLLNEGSSITIQEYSEKINTLEDELIRLQNELEKAKEIDNTRSE
1492'  KDEIDAANNAKAKAERKSKELEVRVA-ELEESLED-KSGTVNVEFIRK-KDAEIDDLRAR
1415'  LEKVSLSNDELLEEKQNTIKSLQDEILSYKDKITRNDKLLSIERDNKR--DLESKQEQ
1549'  LDRETESRIKSDPKNTRKQFAD---LEAKVEEAQREVVTIDRLKLLKLESDIIDLSTQ
1472'  LRAAQESKAKVEEGLKLEEE-SKKEAELEKSKEMMKLESTIESNETELKSSMETIR-
1605'  LDTETKSRIKIEKSKKLEQLTAEERRAAEAGSSKADEEIRKQVWQEVDELRAQLDSERA
1530'  --KSDEKLEQSKSABEDIK-NLQHE---KSDLISRINESEKDEIELKSKLRIEAKSGSE
1665'  SLNASEKIKSLVAEVDVEKQLEDEILAKDKLVKAKRALEVELEEVDRQLEEEEDSRSE
1584'  LETVKQELNNAQEKIRINAENTVLKSKLEDIERELKDKQAEIKSNQEEKELLTSRLKEL
1725'  LEDSKRRLTTEVEDIKKYDAEVEQNTKLEAKKLLTDDVDTLK-KQLEDE---KKKLNES
1644'  EQELDSTQQAQKSEERRAEVR-KFQVEKSLQDEKAMLETKYNDLVNKEQAWKRDEDT
1782'  ERAKRLSEENEDFLAKLDPAEVNRSRAEKDRKRYEYDLKDTKYK-LNDEAATKQTQTEIG
1703'  VKKTTDSQRQEI EKLAELDNLKAENSKLKEANEDRSEIDDLMLLVTDLDEKNAKYSKL
1841'  AAKLEDQIDELRSKLEQEQAK-ATQADKSKKTLEG--EIDNLRQIEDEGKIKMRLEEK
1763'  KDLGVEISSDEEDDEDEEDEEGQVA
1898'  RALEGELBELRETVEEAEDSKSEAEQSKRLVELELEDARRNLQKEIDAKEIAEDAKSNLQ

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Figure 8. Comparison of the predicted amino acid sequence of Usolp protein and myosin heavy chain of slime mold. The yeast sequence has been aligned to give optimal homology; identities of amino acids are indicated by asterisks. These sequence data are available from EMBL Gen/Bank/DBJ under accession number X54378.

and the survival of the *usol* cells was only 30% after 2 h incubation at 37°C (data not shown). This may suggest that the expression of the *usol* mutation causes irreversible damages. We observed the abnormal morphology of microtubules in

the nucleus and bundles of microfilaments in the cytoplasm of the *usol* cells. In the eukaryotic cells, formation of transport vesicles from the ER is the first step of membrane traffic, and vesicles have to be transported effectively and precisely to the next organelle. These dynamic processes may require cooperation with cytoskeletal components.

There are three major cytoskeletal components in the eukaryotic cells: actin filaments, microtubules, and intermediate filaments. Recently, several experiments have been reported in which microtubules are suggested to be involved in some membrane trafficking steps (Kelly, 1990). In the secretory pathway, stabilization of microtubules is required for transport of the newly synthesized proteins from the *trans*-Golgi network to specific secretory vesicles (Kreis et al., 1989), and for fusion of the endocytotic transport vesicles with prelysosomal compartments (Gruenberg et al., 1989). Depolymerizing microtubules by antibiotics also inhibits the flow of the 53-kd protein, unique salvage compartment antigen, from the salvage compartment to the ER (retrograde), but not from the ER to the Golgi complex (anterograde) (Lippincott-Schwartz et al., 1990). Kinesin and dynein are microtubule based mechanochemical ATPases and cause the vesicular transport along microtubules. On the other hand, conditional actin mutant, *act1*, shows a partial defect of secretion and accumulation of secretory vesicles (Novick and Botstein, 1985). It suggests that actin plays a supporting role in secretion especially in the exocytotic step. Relationships between intracellular protein transport and intermediate filaments are unknown. The components of intermediate filaments have a common domain structure: the head domain containing many Arg, Gly, Ser residues; the rod domain of α -helical coiled-coil structure; and the tail domain (Steinert and Roop, 1988; Geisler and Weber, 1986). The cytoskeletal components which play direct roles in the protein transport from the ER to the Golgi apparatus are unknown to date.

We have cloned the wild type *USO1* gene by complementation of the temperature-sensitive growth of the *usol* mutant. DNA sequence analysis demonstrates that *USO1* encodes a large protein consisting of two distinct domains: NH₂-terminal 700 amino acids with an unknown function, and COOH-terminal 1,100 amino acids which form an α -helical rod. Several filamentous proteins that contain α -helical coiled-coil regions have characteristic 7-amino acid repeats, and hydrophobic residues are strongly biased at the first and fourth positions (McLachlan and Karn, 1983). Usolp COOH-terminal domain clearly shows this feature, especially in leucine residues (Fig. 9B), indicating that Usolp forms a coiled-coil structure. The *USO1* gene is essential for growth, but the *usol-1* mutation can be complemented by the *USO1* gene without its COOH-terminal 800 amino acids. Minimal functional gene which has the complementation activity for *usol* mutant has only a 300-amino-acid long α -helical domain. Sec2p is previously reported to have an α -helical coiled-coil domain, and is required for the post-Golgi stage of the secretory pathway. The COOH-terminal half of Sec2p is dispensable for growth, but the NH₂-terminal half, containing coiled-coil domain, is essential for growth (Nair et al., 1990). The domain structure and functional point of Sec2p are quite different from those of Usolp.

We found a consensus sequence of Ca²⁺-binding loops in the hyperhydrophilic region (position 469–487) of Usolp (Fig. 5); Usolp function may be regulated by Ca²⁺. Yeast

A

	abcdefg		abcdefg		abcdefg
750	QTETEST	1105	LETSEKA	1458	ERDNKRD
	HENLTEK		LKEVKEN		LESLKEQ
	LIALTNE		EEHLKER		LRAAQES
	HKELDEK		KIQLEKE		KAKVEEG
	YQILNNS		ATETKQQ		LKKLEEE
	HSSLKEN		LNSLRAN		SSKEKAE
	FSILETE		LESLEKE		LEKSKEM
	LKNVRDS		HEDLAAQ		MKKLEST
	LDEMTQL		LKKYEEQ		IESNETE
	RDVLETK		IANKERQ		LKSSMET
820	DKENQTA	1175	YNEEISQ	1528	IRKSDEK
	LLEYKST		LNDEITS		LEQSKKS
	IHKQEDS		TQQENES		AEDDIKN
	IKTLEKG		IKKKNDE		LQHEKSD
	LETILSQ		LEGEVKA		LISRINE
	KKKAEDG		MKSTSEE		SEKDIEE
	INKMGKD		QSNLKKSE		LKSKLRI
	LFALSRE		IDALNLQ		EAKSGSE
	MQAVEEN		IKELKKK		LETVKQE
	CKNLQKE		NETNEAS		LNNAQEK
890	KDKSNVNHQKE	1246	LLESIKS	1598	IRINAEE
	TKSLKED		VESETVK		NTVLKSK
	IAAKITE		IKELQDE		LEDIERE
	IKAINEN		CNFKEKE		LKDKQAE
	LEEMKIQ		VSELEDK		IKSNQEE
	CNNLSKE		LKASEDK		KELLTSR
	KEHISKE		NSKYLEL		LKELEQE
	LVEYKSR		QKSESEKI		LDSTQOK
	FQSHDNL		KEELDAD		AQKSEEE
	VAKLTEK		TTELKIQ		RRAEVRK
964	LKSLANN	1316	LEKITNL	1668	FQVEKSO
	YKDMQAE		SKAKEKS		LDEKAML
	NESLIKA		ESELSRL		LETKYND
	VEESKNE		KKTSSEE		LVNKEQA
	SSIQLSN		RKNAEEQ		WKRDEDT
	LQNKIDS		LEKLNKE		VKTTTDS
	MSQEKEN		IQIKNOA		QRQIEIK
	FQIERGS		FEKERKL		LAKELDN
	IEKNIEQ		LNEGSST		LKAENSK
	LKKTISD		ITQYSEK		LKEANED
1034	LEQTKEE	1387	INTLEDE	1738	RSEIDDL
	IISKSDS		LIRLQNE		MLLVTDL
	SKDEYESQ		NELKAKE		DEKNAKY
	ISLLKEK		IDNTRSE		RSKLKDL
	LETATTA		LEKVSLs		GVEISSD
	NDENVNK		NDELLEEK		EEDDEED
	ISELTKT		QNTIKSL		DEEDEEE
	REELEAE		QDEILSY		QQVA
	LAAYKNL		KDKITRN		
	KNELETK		DEKLLSI		

B

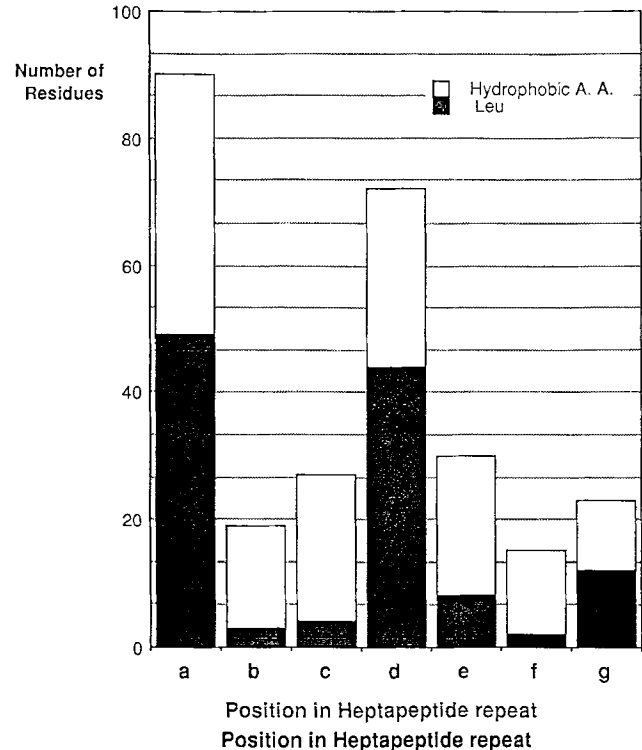


Figure 9. Seven amino acid repeats of Usolp sequence. (A) shows the region of heptapeptide repeats found in the COOH-terminal half region of Usolp beginning from Gln 750. The numbers on the left indicate the residue number of these amino acids with respect to the protein sequence. The sequence is arranged in seven-residue repeats in each row with five exceptions. The positions a, b, c, d, e, f, and g are marked above the column. B is the histograms that show the distributions of hydrophobic residues. Closed bars indicate the numbers of leucine residues.

Pmrl gene, which is identical to *SSCI*, encodes a Ca^{2+} -ATPase and the *pmrl* mutant cells secrete proteins lacking outer chain carbohydrate (Rudolph et al., 1989). Predicted *Pmrlp* sequence has a Ca^{2+} -binding loop-related structure which is present in a variety of Ca^{2+} -binding/regulatory proteins such as calmodulin (Kretsinger, 1980). Usolp has no homologous sequence to *Pmrlp* or calmodulin but position 475-487 (DKDIDGKDGTEYE) matches the consensus sequence of Ca^{2+} -binding loop (DxDxDGxxxxxE) characteristic of the EF hand (Davis et al., 1986; Rudolph et al., 1989).

Usolp has another stretch of 15 acidic residues at the COOH-terminal region (Fig. 6, position 1,772-1,786). The role of the stretch is unknown. Consecutive acidic amino acid residues

have been found in some nuclear proteins (nucleoplasm, nucleolin, high mobility group 1:HMG-1, centromere protein B), and structural roles have been proposed for these domains (Earnshaw, 1987). The *SEC7* gene is required for protein transport from the Golgi apparatus to the secretory vesicles and putative *Sec7p* also contains stretches of 13 and 14 consecutive glutamate/asparatate residues (Achstetter et al., 1988). These domains may play an important role in regulation of *Sec7p* or interaction with other components of intracellular membrane traffic.

The function of Usolp in the intracellular protein transport is unknown but several possibilities can be discussed. First, Usolp may be a kinesin-like motor protein to drive the vesicular transport from the ER to the Golgi. Motor proteins are

considered to generate driving energy by hydrolysis of ATP, but we found no typical ATP binding region consensus sequence (GxxxxGKTxxxxxI/V) (Walker et al., 1982) in the Usolp polypeptide. If Usolp is related to a motor, another energy-supplying subunit may be required for the transport. Second, Usolp may be a cytostructural protein to enclose transport vesicles in a lattice structure like a clathrin coat. Sec7p, which has similar molecular weight with Usolp and stretch of acidic residues, could serve as a coat protein of Golgi stacks. Investigation of the intracellular localization and membrane association of Usolp will give information for the function of Usolp. Third, Usolp may itself form a cytoskeletal structure along which transport vesicles are driven from the ER to the Golgi apparatus. The long α -helical domain of Usolp suggests that Usolp exists as a coiled-coil and might form a filamentous structure in the cell. Further biochemical analysis involving purification and in vitro polymerization and depolymerization of Usolp should be required to examine this possibility. We also have to consider the possibility that the function of Usolp is indirect in vesicular transport. We observed that it took 20–30 min incubation at 37°C to express the transport block, and membrane organelles were drastically changed in morphology. The loss of Usolp function may affect the structure of organelles and cause the block of intracellular membrane traffics. To elucidate the role of Usolp in the secretory pathway, it will be necessary to search for the factors cooperating with Usolp by genetic and biochemical approaches.

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