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Identification of 23 Complementation Groups Required for Post-translational Events in the Yeast Secretory Pathway

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Summary

Cells of a Saccharomyces cerevisiae mutant that is temperature-sensitive for secretion and cell surface growth become dense during incubation at the nonpermissive temperature (37°C). This property allows the selection of additional secretory mutants by sedimentation of mutagenized cells on a Ludox density gradient. Colonies derived from dense cells are screened for conditional growth and secretion of invertase and acid phosphatase. The *sec* mutant strains that accumulate an abnormally large intracellular pool of invertase at 37°C (188 mutant clones) fall into 23 complementation groups, and the distribution of mutant alleles suggests that more complementation groups could be found. Bud emergence and incorporation of a plasma membrane sulfate permease activity stop quickly after a shift to 37°C. Many of the mutants are thermoreversible; upon return to the permissive temperature (25°C) the accumulated invertase is secreted. Electron microscopy of *sec* mutant cells reveals, with one exception, the temperature-dependent accumulation of membrane-enclosed secretory organelles. We suggest that these structures represent intermediates in a pathway in which secretion and plasma membrane assembly are colinear.

Introduction

Studies of the secretory process in eucaryotic cells have focused on the molecular events associated with synthesis and processing of specific secretory and membrane proteins, and on the organelles which mediate passage from the endoplasmic reticulum to the cell surface. While much is known about the maturation of certain secretory and membrane polypeptides (such as insulin, Chan, Keim and Steiner, 1976; VSV glycoprotein, Katz et al., 1977), the molecular events associated with sorting, packaging, transport and exocytosis of the exported proteins remain obscure.

We have undertaken a study of the secretory apparatus in the yeast Saccharomyces cerevisiae. While yeast cells are not specialized for secretion as are, for example, the acinar cells of the pancreas (Palade, 1975), the ease of a combined genetic and biochemical approach allows the use of techniques which have been less feasible with traditional secretory systems. The use of conditional mutants has been crucial for the analysis of complex bacteriophage morphogenesis pathways, both in identifying intermediate structures and in providing biochemical assays for assembly steps (Wood and King, 1979). We believe that a similar approach may be useful in unraveling a eucaryotic morphogenesis pathway.

Yeast cell surface growth is restricted primarily to enlargement of the bud followed by cell division. Incorporation of new cell wall material, including secretion of the wall-bound enzymes invertase and acid phosphatase, is also restricted to the bud (Tkacz and Lampen, 1972, 1973; Field and Schekman, 1980). Membrane-enclosed vesicles have been implicated in secretion and bud growth (Moor, 1967; Matile et al., 1971). Our recent report of a conditional mutant blocked in secretion and cell surface growth, which accumulates membrane-enclosed vesicles containing a secretory enzyme (Novick and Schekman, 1979), supports such a role for vesicles.

In this report we describe a technique for the enrichment of conditional secretory and cell surface growth mutants. We have identified a large number of complementation groups that are required for the movement of at least two secretory enzymes and one plasma membrane permease through a series of distinct membrane-enclosed organelles in a pathway that leads to the cell surface.

Results

Secretory mutants are defined as those strains which fail to export active invertase and acid phosphatase, but continue to synthesize protein under restrictive growth conditions. In a previous report (Novick and Schekman, 1979) we described a screening procedure that allowed the identification of two nonallelic secretory mutants (*sec*1-1, *sec*2-1) among a group of randomly selected temperature-sensitive yeast mutants. The first mutant (HMSF 1) stopped dividing and enlarging at the nonpermissive temperature (37°C), yet protein and phospholipid synthesis continued for at least 3 hr. This situation produced dense cells. Henry et al. (1977) showed that during inositol starvation of an auxotrophic strain, net cell surface growth stopped while cell mass increased. Starved cells could be resolved from normal cells on a Ludox density gradient. We have used the Ludox density gradient technique to select additional secretory mutants.

Density Enrichment

In the experiment shown in Figure 1, about 5×10^6 *sec*1-1 cells were mixed with 5×10^8 X2180 cells, and after 3 hr at 37°C the mixture was sedimented in a solution of Ludox. The resulting gradient was fractionated and the genotype of cells in diluted aliquots was determined. The 5% increase in density allowed *sec*1 cells to be separated completely from a 100 fold larger population of wild-type cells.

The density separation made feasible the isolation

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of a large number of additional secretory mutants. Mutagenized cultures were allowed to grow for several generations at 24°C and then transferred to 37°C for 3 hr. The cells were then sedimented in a Ludox gradient, and the densest 1–2% of the cells were pooled. Temperature-sensitive growth mutants were identified among the dense cells, and the secretion of acid phosphatase and invertase was measured by a modification of the previous procedure (Novick and Schekman, 1979). The density gradient procedure enriched for a variety of temperature-sensitive mutants and among them about 15% were secretion-defective (Table 1).

The procedure was used on three strains; NF1R and SF182-3B were mutagenized with ethyl methanesulfonate (EMS) and X2180-1A was treated with nitrous acid. A total of 485 secretion-defective mutants were isolated, among which three classes were found. Class A *sec* mutants (188 total) showed accumulation of invertase at the nonpermissive temperature. Class B *sec* mutants showed no accumulation of active invertase, although protein synthesis continued at a high rate at 37°C. Class C mutants did not secrete because protein synthesis was temperature-sensitive. This report will deal with the *sec*A mutants; analysis of the *sec*B mutants is in progress.

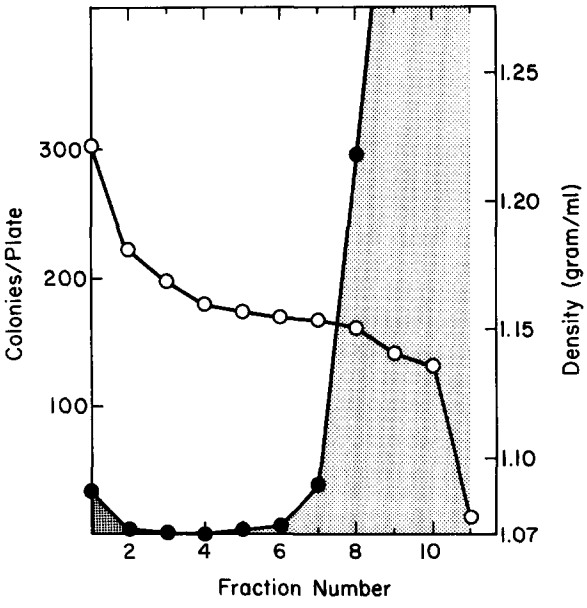

Figure 1. Density Gradient Separation of *sec*1-1 and X2180 Cells

SF150-5C and X2180-1A cells were grown in YPD medium at 25°C. After 3 hr at 37°C, the cells were sedimented, washed and resuspended in 1 ml of water. Cell aliquots were mixed (49.5 A_{600} units of X2180 and 0.5 A_{600} unit of SF150-5C) and sedimented on a Ludox gradient. Fractions were collected and diluted 10^4 fold, and 0.1 ml portions were spread on minimal medium (7 mM phosphate) agar plates. Colonies formed after 2 days at room temperature were stained for acid phosphatase activity (Hansche et al., 1978). Heavy stippling represents the phosphatase-constitutive (*pho*80) *sec*1 colonies (SF150-5C); light stippling represents phosphatase-repressed colonies (X2180).

The enrichment procedure was first performed on an *a* strain (NF1R) and then on an α strain (SF 182-3B). The *sec* mutants were arranged into complementation groups by standard genetic techniques. The enrichment was repeated with strain X2180-1A, and complementation analysis of the temperature-sensitive clones was performed with tester strains derived from the previously isolated *sec* mutants. New *sec* mutants that complemented all of the tester strains were crossed with X2180-1B, diploids were selected and sporulated, and the *sec* mutants were obtained in *a* and α mating type strains. Complementation analysis was performed again. By this procedure, 23 complementation groups were identified. EMS and nitrous acid produced a similar spectrum of mutant alleles (Table 2); *sec*2 and *sec*5 were the most common groups for each mutagen.

All the *sec* mutants were recessive in heterozygous diploids. Analysis of each complementation group was conducted with a representative allele chosen for optimum growth at 25°C, maximum inhibition of secretion at 37°C and maximum secretion of accumulated invertase upon return to 25°C. Each group showed 2:2 segregation of the temperature-sensitive phenotype which always coincided with the secretory defect.

Thermoreversible Accumulation of Invertase

External invertase synthesis is derepressed by a decreased supply of glucose, and the secreted enzyme remains in the yeast cell wall and can be assayed in a whole-cell suspension (Dodyk and Rothstein, 1964). Intracellular forms of the enzyme are measured in a spheroplast lysate, and this level does not rise significantly during derepression (Novick and Schekman, 1979).

The level of secreted invertase increased 13 fold when X2180 cells were transferred from YP + 5% glucose medium to YP + 0.1% glucose medium (Table 3). This increase was blocked to varying degrees in the *sec* mutants at 37°C. Derepression for 1 hr at 37°C produced a 4–18 fold increase in the intracellular level of invertase. Upon return to 25°C, in the

Table 1. Comparison of Screening Procedure with and without Density Enrichment

	Without Enrichment		With Enrichment	
Screening Stage	Colonies	%	Colonies	%
(1) Colonies tested	5,600	100	18,500	100
(2) TS mutants	291	5.2	2,830	15
(3) TS phosphatase secretion	63	1.1	980	5
(4) TS invertase secretion	16	.29	485	2.6
(5) TS invertase accumulation	2	.04	188	1.0

Table 2. Distribution of Mutants in the *sec*A Complementation Groups: EMS versus Nitrous Acid

	EMS		Nitrous Acid	
sec	Isolates	%	Isolates	%
1	8	11	4	3
2	28	39	41	35
3	3	4	0	0
4	7	10	2	2
5	10	14	16	14
6	3	4	3	3
7	1	1	3	3
8	6	8	4	3
9	3	4	4	3
10	1	1	2	2
11	1	1	11	9
12	1	1	3	3
13			4	3
14			4	3
15			2	2
16			2	2
17			1	1
18			2	2
19			1	1
20			1	1
21			1	1
22			4	3
23			1	1

presence of cycloheximide, all the mutants showed an increased level of secreted invertase. In most cases this represented the secretion of a large fraction of the accumulated enzyme. The mutants produced nearly normal levels of secreted and intracellular invertase when derepression was conducted at 25°C.

All 12 alleles of *sec*11 produced nearly normal levels of secreted invertase at 37°C. However, like the other mutants, *sec*11 accumulated about 7 fold more internal invertase than normal. Furthermore, 35% of the accumulated enzyme was secreted upon return to 25°C.

Other Defects

Secreted acid phosphatase first appeared in wild-type cells 1.5 hr after a transfer from YPD + 7 mM phosphate medium into phosphate-depleted YPD medium, and the rate of secretion was maximal from 2.5 to 5 hr after the shift. During this 2.5 hr period, the *sec* mutants secreted normally at 25°C, but produced at least 5 fold less external phosphatase at 37°C (Table 4).

Incorporation of a sulfate permease activity was used to assess the role of the *sec* gene products in plasma membrane assembly. In X2180, sulfate permease activity first appeared 30 min after cells were transferred from a minimal medium containing 1.5 mM methionine to a sulfate-free minimal medium. During a 2.5 hr period of derepression, most of the *sec* mutants produced normal levels of permease activity at 25°C, but showed significantly lower incorporation at 37°C (Table 4).

Bud emergence stopped quickly, as indicated by the nearly constant number of cells and buds, when the *sec* mutants were transferred from 25° to 37°C (Table 4). Cells arrested at all stages of the cell cycle, and no increase in cell size was noted. As expected from the enrichment procedure, all the *sec* mutants became denser than X2180 during a 3 hr incubation at 37°C (Table 4), although only a few of the strains became as dense as *sec*1. Certain other conditions, such as inhibition of protein synthesis or growth to stationary phase, caused X2180 cells to become dense at 37°C.

Mutants Accumulate Secretory Organelles

The reversible accumulation of invertase and the reduced incorporation of a membrane permease suggested that the *sec* mutants might accumulate an organelle of the secretory apparatus. This was confirmed for all but one of the mutants when thin sections were examined by electron microscopy. Wild-type cells grown at 37°C (Novick and Schekman, 1979, Figure 6A) or *sec* mutant cells grown at 25°C (Figure 2A) showed occasional enrichment of vesicles in the bud; short, thin tubules of endoplasmic reticulum (ER) were also seen apposed to the inner surface of the plasma membrane or in continuity with the nuclear membrane. Mutant cells incubated for 2 hr at 37°C showed several cytological aberrations. The groups were classified according to the organelle accumulated. The most common class, with 10 members (Table 5, Figure 2B), accumulated membrane-enclosed vesicles of 80–100 nm in diameter. These vesicles were not enriched in the bud.

A second class, with nine representatives, developed a more extensive network of ER than was seen in wild-type cells (Figure 3). The ER often lined the inner surface of the plasma membrane and wound through the cytoplasm where multiple connections with the nuclear membrane were visible (Figures 3A and 3B). The lumen of both the ER and the nuclear membrane was wider than the corresponding wild-type structure. Eucaryotic rough and smooth ER can be distinguished by the presence or absence of attached ribosomes. However, due to the high concentration of free ribosomes, it was not possible to identify specific associations with the yeast ER. While all nine members of this class showed extensive ER at 37°C, three complementation groups also produced small vesicles (~ 40 nm) which were often arranged in patches in the cytoplasm (Table 5, Figure 3C).

Table 3. Invertase Secretion and Accumulation by the *sec* Mutants

Strain	*sec* Group	Units/mg Dry Weight: External[a] (1 Hr 37°C)	Internal (1 Hr 37°C)	External (1 Hr 37°C → 3 Hr 25°C)	% Release[b]	External (1 Hr 25°C)	Internal (1 Hr 25°C)
X2180-1A		.38	.08	.33	0	.34	.14
HMSF 1	1-1	.02	.61	.28	43	.29	.15
HMSF 106	2-56	.03	.87	.36	38	.24	.18
HMSF 68	3-2	.02	.31	.05	9	.31	.22
HMSF 13	4-2	.05	.63	.13	11	.32	.30
HMSF 134	5-24	.03	.84	.08	6	.39	.17
HMSF 136	6-4	.03	.84	.46	52	.36	.14
HMSF 6	7-1	.04	.39	.10	16	.42	.29
HMSF 95	8-6	.03	.57	.07	7	.37	.22
HMSF 143	9-4	.09	1.05	.53	42	.20	.28
HMSF 147	10-2	.03	.68	.15	18	.31	.17
HMSF 154	11-7	.40	.53	.59	35	.56	.26
HMSF 162	12-4	.04	1.3	.90	64	.22	.11
HMSF 163	13-1	.19	.77	.64	58	.28	.14
HMSF 169	14-3	.07	.54	.32	46	.30	.12
HMSF 171	15-1	.17	.47	.33	34	.36	.19
HMSF 174	16-2	.06	1.50	.69	42	.45	.18
HMSF 175	17-1	.17	.70	.58	59	.29	.14
HMSF 176	18-1	.02	.97	.64	63	.36	.15
HMSF 178	19-1	.02	1.05	.49	45	.43	.21
HMSF 179	20-1	.07	.98	.68	63	.49	.24
HMSF 180	21-1	.29	.43	.48	44	.39	.18
HMSF 183	22-3	.04	.84	.58	64	.41	.18
HMSF 190	23-1	.03	.83	.57	65	.36	.15

[a] Cultures were grown overnight in YP + 5% glucose medium at 25°C to an A_{600} of 0.5–5.5. Cells (3 A_{600} units) were sedimented in a clinical centrifuge, resuspended in 3 ml of YP + 0.1% glucose medium and incubated at 37°C for 1 hr. An aliquot (1 ml) was then removed and added to a tube containing 1 mg of glucose and 0.1 mg of cycloheximide, and the mixture was incubated at 25°C for 3 hr. In a parallel experiment, 2 A_{600} units of the overnight culture were sedimented, and the cell pellet was resuspended in 2 ml of YP + 0.1% glucose medium and incubated at 25°C for 1 hr. At the end of each experiment samples were chilled on ice, centrifuged and resuspended in one half volume of 10 mM azide at 0°C.

[b] $\%\ \text{release} = \left(\frac{\text{Ext}_{37°C \rightarrow 25°C} - \text{Ext}_{37°C}}{\text{Int}_{37°C}}\right) 100.$

A third class of mutant produced an organelle with no obvious counterpart in other eucaryotic cells. Because of its unique morphology, we call this structure a Berkeley body (Bb). The Bb, although varied in form, appeared to consist of two curved membranes with an enclosed electron-transparent lumen (Figure 4). In some sections the Bb was closed to form a toroid; in other sections it was open at one end to form a cup (Figure 4B). The toroid structure, with enclosed ribosomes and cytoplasm, may be an alternate view of the cup form; perpendicular planes of sectioning would give the image of one or the other. Two complementation groups (*sec*7, 14) made predominantly Bbs; two alleles of *sec*7, derived from different parent strains, were examined and each produced only Bbs. In addition to Bbs, *sec*14 also produced 80–100 nm vesicles (Figure 5A). Bbs were occasionally seen in sections of two complementation groups (*sec*2, 9) where 80–100 nm vesicles were the dominant structure.

Two exceptions to the major classes were observed: *sec*19 produced a mixture of the major organelles (Figure 5B), and *sec*11 did not build up any of the organelles.

Discussion

The results presented here show that in yeast, at least 23 gene products are required for the transport of secretory proteins from the site of synthesis to the cell

Table 4. Acid Phosphatase Secretion, Sulfate Permease Incorporation, Cell Division and Cell Density of the *sec* Mutants

Strain	*sec*	Acid Phosphatase[a] (Units/ml)			Sulfate Permease[b] $\left(\frac{\text{Units}}{\text{mg Dry Weight}}\right)$		Cell Number[c] $\left(\frac{\text{2 Hr 37°C}}{\text{0 Hr}}\right)$	Cell Density[d] (g/ml)	
		2.5 Hr	5 Hr 37°C	5 Hr 25°C	25°C	37°C		25°C	37°C
X2180 1A		27	193	174	6.3	5.0	2.03	1.110	1.122
HMSF 1	1-1	27	28	147	7.4	.2	1.10	1.113	1.161
HMSF 106	2-56	50	48	170	5.8	.1	.92	1.109	1.141
HMSF 68	3-2	55	82	411	6.8	.3	1.10	1.109	1.142
HMSF 13	4-4	25	29	202	4.8	.2	1.07	1.116	1.146
HMSF 134	5-24	27	31	177	5.8	.4	1.04	1.110	1.161
HMSF 136	6-4	25	27	178	5.1	.1	1.20	1.111	1.159
HMSF 6	7-1	86	95	320	3.6	.03	.91	1.103	1.142
HMSF 16	8-1	44	81	223	4.5	.9	1.11	1.103	1.135
HMSF 143	9-4	30	30	177	6.1	.1	1.04	1.111	1.146
HMSF 147	10-2	25	47	70	7.2[e]	.03	.92	1.117	1.152
HMSF 154	11-7	16	15	107	6.1	2.0	1.48	1.117	1.142
HMSF 162	12-4	25	25	89	5.3[e]	.65	1.05	1.107	1.143
HMSF 163	13-1	22	18	154	4.2	.1	1.01	1.113	1.141
HMSF 169	14-3	25	26	121	5.3	1.5	.93	1.117	1.144
HMSF 171	15-1	23	41	190	6.7	1.7	1.15	1.117	1.159
HMSF 174	16-2	26	25	93	6.2	.02	.95	1.114	1.139
HMSF 175	17-1	32	30	200	6.1	.4	1.07	1.118	1.146
HMSF 176	18-1	27	25	202	5.6	.04	.93	1.117	1.155
HMSF 178	19-1	32	32	237	6.3	.3	.92	1.119	1.156
HMSF 179	20-1	23	21	136	1.5	.1	.94	1.116	1.141
HMSF 180	21-1	24	42	171	6.3	.2	1.08	1.113	1.142
HMSF 183	22-3	25	26	204	1.7	.1	1.09	1.115	1.151
HMSF 190	23-1	30	27	82	4.4	.1	1.02	1.114	1.145

[a] Cultures were grown overnight in YPD + 7 mm phosphate medium. Cells (4.5 A_{600} units) were centrifuged, resuspended in 3 ml of phosphate-depleted YPD medium and incubated at 25°C. After 2.5 hr, 1 ml aliquots were transferred to 37° and 0°C, and the rest were left at 25°C. Incubation was continued for 2.5 hr and the samples were chilled, centrifuged and resuspended in 1 ml of 10 mM azide at 0°C.

[b] Cultures were grown overnight in minimal medium + 1.5 mM methionine and 50 μM $(NH_4)_2SO_4$. Cells (1.2 A_{600} units) were centrifuged, washed once and resuspended in 1.2 ml of sulfate-free minimal medium. After 2.5 hr at 25° or 37°C, the tubes were chilled and 1 ml aliquots were removed for the permease assays; the rest were used for A_{600} determination.

[c] Cultures were grown overnight in YPD medium at 25°C. Cells (2 A_{600} units) were centrifuged and resuspended in 2 ml YPD medium; 0.5 ml was diluted with 10 mM azide at 0°C and the rest were incubated for 2 hr at 37°C. The ratio of the 2 hr/0 hr cell number is listed.

[d] Cultures were grown overnight in YPD medium. Cells (8 A_{600} units) were centrifuged and resuspended in 4 ml of YPD medium; 2 ml of each were incubated at 25° and 37°C. After 3 hr the cells were sedimented and resuspended in 0.5 ml of 10 mM azide at 0°C. X2180 cells grown to stationary phase at 37°C had a density of 1.131 g/ml; cells treated with 0.1 mg/ml of cycloheximide for 3 hr at 37°C had a density of 1.134 g/ml.

[e] HMSF 147 and 162 were auxotrophic and the sulfate permease experiment was carried out with prototrophic strains derived from crosses with X2180 (SF 226-1C, *sec*10; SF 292-2C, *sec*12).

surface. Thermosensitive defects in these gene products also block incorporation of a plasma membrane permease and stop bud growth. Taken together, these observations suggest that membrane growth and secretion are accomplished by parallel if not identical pathways. Furthermore, membrane-enclosed organelles accumulate in 22/23 of the mutants at 37° but not at 25°C. We propose that these structures are intermediates in the secretory pathway; their soluble contents are destined for secretion by exocytosis and their membranes will be incorporated into the plasma membrane by fusion.

In a previous report (Novick and Schekman, 1979), we described the detection of *sec*1-1 and *sec*2-1 in a collection of randomly selected temperature-sensitive mutants. No new *sec* mutants were found in a larger collection of temperature-sensitive strains, and therefore the density enrichment procedure was adopted. Although both of the original *sec* mutants become dense at 37°C and survive the enrichment, density

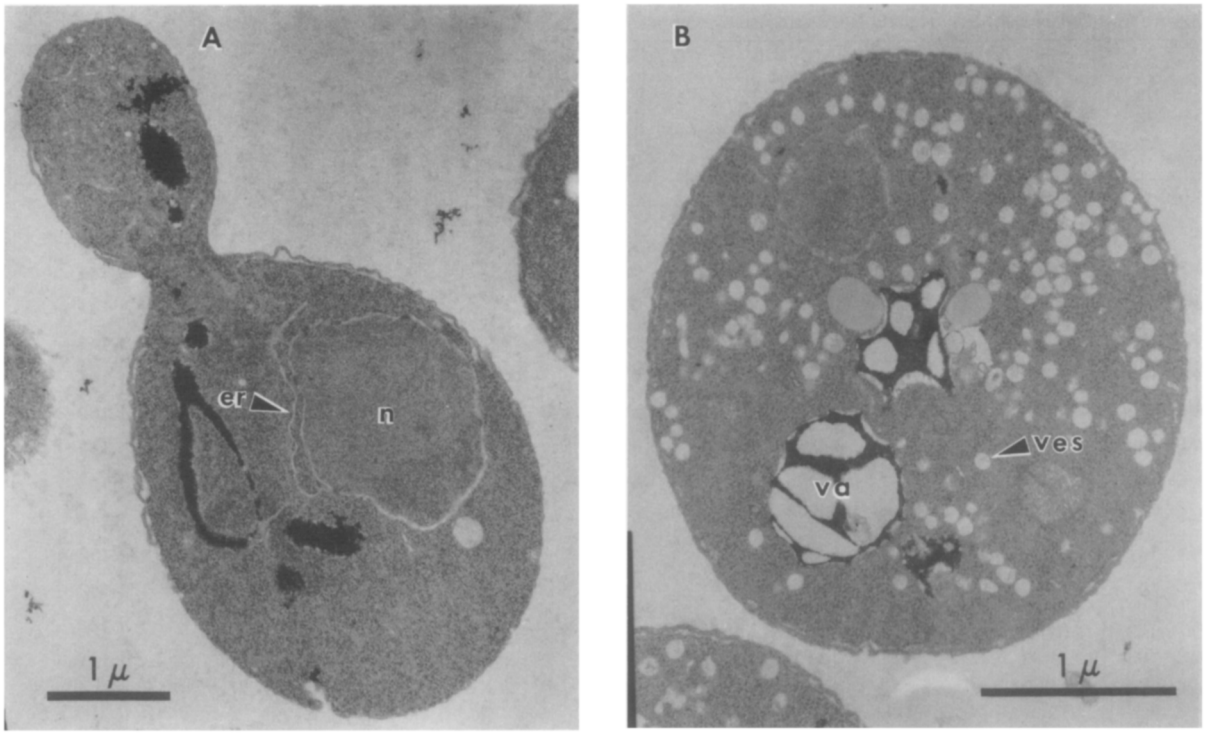

Figure 2. Thin Section Electron Micrographs of Cells Grown in YPD Medium

(A) HMSF 13 (*sec*4-2) grown at 25°C; (B) HMSF 171 (*sec*15-1) incubated at 37°C for 2 hr. Symbols: (n) nucleus; (va) vacuole; (er) endoplasmic reticulum; (ves) vesicles.

selection has the disadvantage that it eliminates mutants that die rapidly at 37°C. We assume that the density enrichment selects mutants that accumulate mass without a corresponding cell surface increase. The screening procedure, on the other hand, assumes only that secretion of cell wall mannoproteins is necessary for cell viability. For this reason, the density enrichment may eliminate mutants which fail to secrete but continue to expand, while the screening procedure will remove mutants which fail to enlarge but continue to secrete. Nevertheless, a large number of gene products are implicated in both aspects of cell surface growth.

It is likely that there are more than 23 *sec*A complementation groups required for the secretory process. Five of the groups reported here contain only one mutant allele, suggesting that groups exist for which no mutant has been found. Furthermore, the 23 *sec*A groups may represent fewer than 23 separate gene products; gene clusters coding for multifunctional proteins have been found in fungi and yeast. However, in such circumstances a single mutant frequently appears to fall into two otherwise distinct complementation groups (Fincham, 1977). No example of overlapping *sec* groups was found.

A trivial explanation for the large number of *sec* complementation groups is that mutant forms of various secreted proteins can act as inhibitors and block the passage of other cell surface molecules. Bassford and Beckwith (1979) and Bassford, Silhavy and Beck-

Table 5. Organelles Accumulated in the *sec* Strains

Strain (HMSF)	*sec*	Structure(s)
1	1-1	vesicles, Berkeley bodies
47	2-7	vesicles
3	3-1	vesicles
13	4-2	vesicles
81	5-8	vesicles
12	6-1	vesicles
6	7-1, -2	Berkeley bodies
93	8-4	vesicles
89	9-3	vesicles, Berkeley bodies
147	10-2	vesicles
154	11-7	
162	12-4	ER
163	13-1	ER
169	14-3	Berkeley bodies, vesicles
171	15-1	vesicles
174	16-2	ER
175	17-1	ER, small vesicles
176	18-1	ER, small vesicles
178	19-1	vesicles, Berkeley bodies, ER
179	20-1	ER
180	21-1	ER
183	22-3	ER, small vesicles
190	23-1	ER

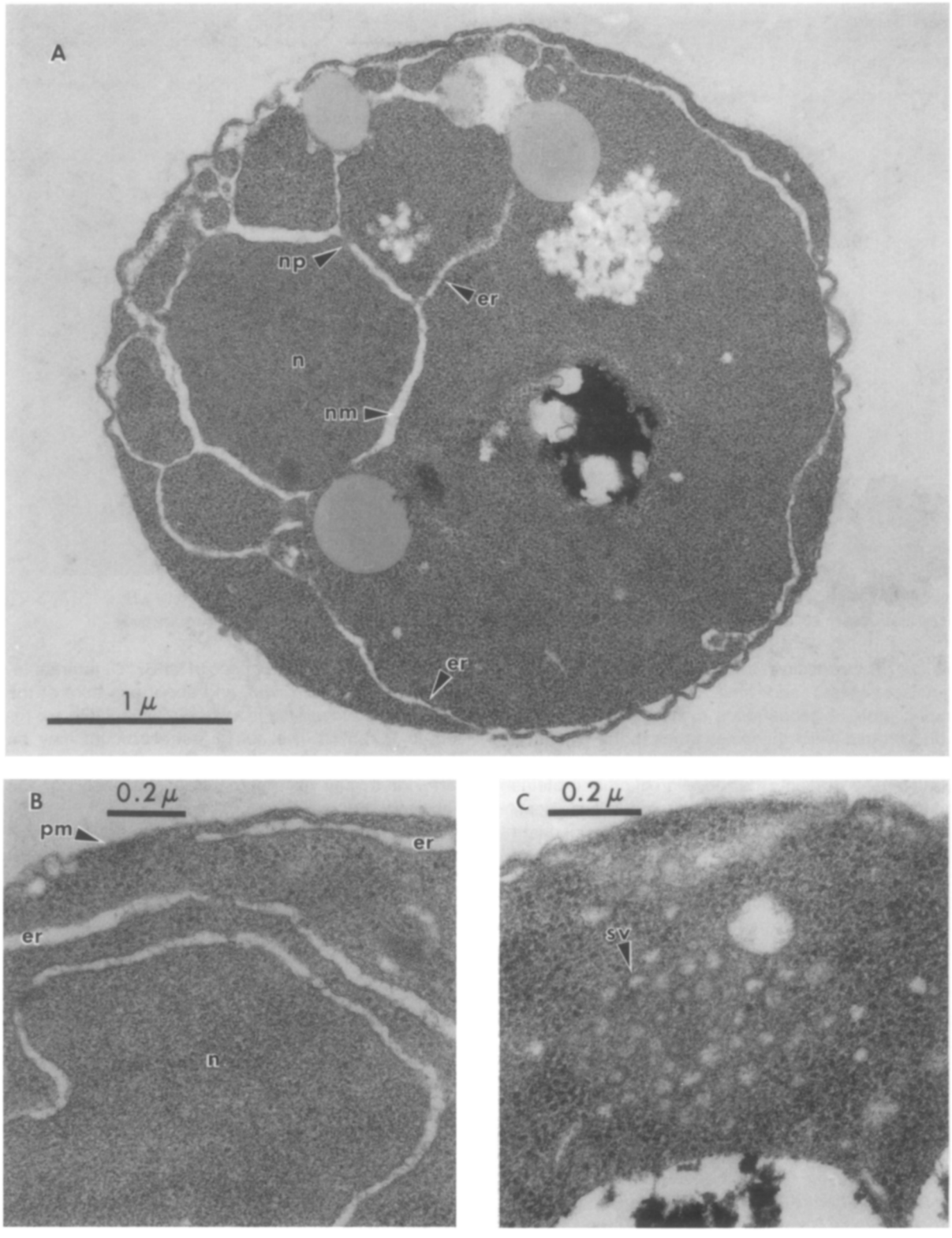

Figure 3. Thin Section Electron Micrographs of Cells Grown in YPD Medium at 25°C, Then Shifted to 37°C for 2 Hr

(A) HMSF 174 (*sec*16-2); (B) HMSF 190 (*sec*23-1); (C) HMSF 175 (*sec*17-1). Symbols are as in Figure 2 and (np) nuclear pore; (sv) small vesicle; (pm) plasma membrane; (nm) nuclear membrane.

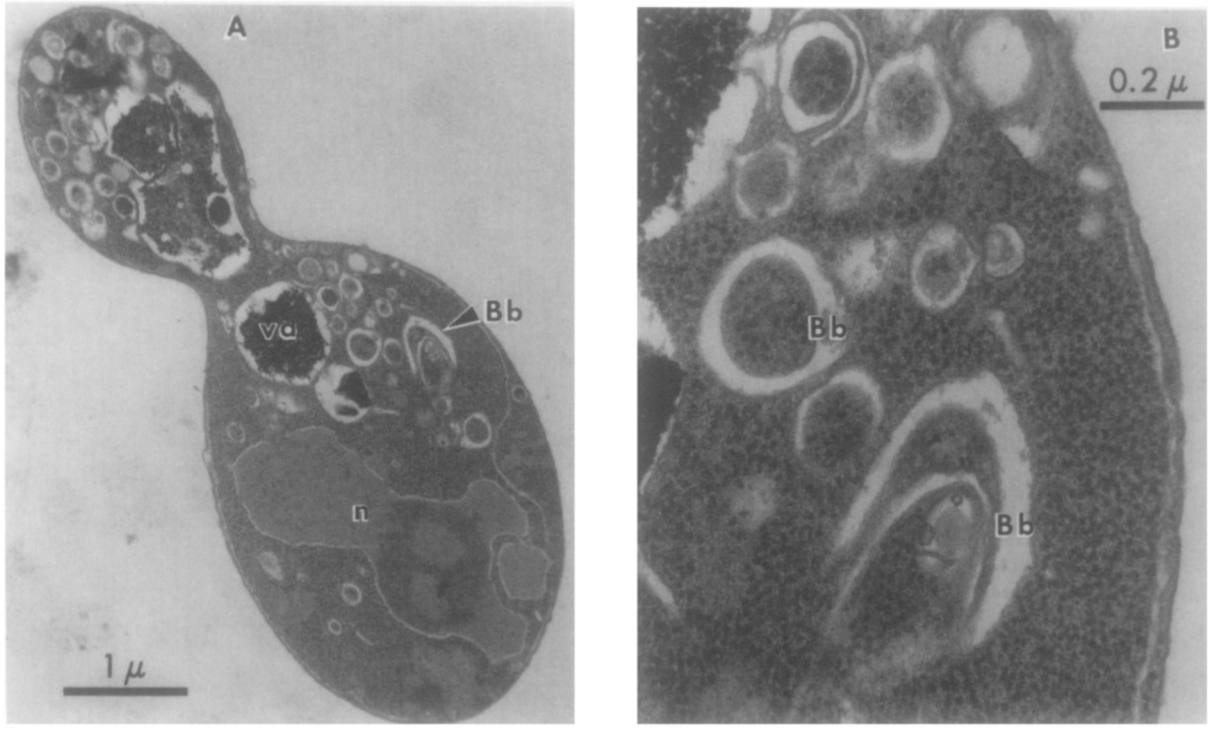

Figure 4. Thin Section Electron Micrograph of HMSF 6 (*sec*7-1) Grown in YPD Medium at 25°C, Then Shifted to 37°C for 2 Hr
(A) Low magnification; (B) a portion of the same cell at higher magnification. Symbols are as in Figure 2 and (Bb) Berkeley body.

with (1979) have shown that fusion of the E. coli *lac*Z (β-galactosidase) and *mal*E (periplasmic maltose binding protein) genes leads to the production of a hybrid protein which becomes stuck in the cytoplasmic membrane. The aberrant incorporation of this hybrid protein prevents the secretion of other proteins and the cells die. Such mutations are genetically dominant (T. Silhavy, personal communication); cell death resulting from the production of hybrid protein is not prevented by synthesis of a normal *mal*E gene product. In contrast, all the *sec* mutants are recessive and therefore the defective gene products are not likely to be secretion inhibitors.

Although 16 of the 23 representative mutant alleles are at least 5 fold reduced at 37°C in each of the four parameters of cell surface growth (Tables 3 and 4), several of the mutants are less restrictive (leaky). Some of the leaky strains (*sec*15, 17, 21) are in complementation groups for which only 1–2 mutant alleles are available. Among the groups with many members, some alleles are very restrictive at 37°C, while others are leaky. Certain strains are less restrictive for one enzyme marker than another. This may be due to the different growth conditions required for derepression of the marker enzymes. Thus while invertase appears in cells within 30 min of a transfer from 5 to 0.1% glucose, acid phosphatase production requires a 1.5 hr phosphate starvation. The *sec*11 group is an extreme example. Cells of all 12 mutant alleles secrete nearly normal amounts of invertase at 37°C, but secrete no acid phosphatase. Although no organelles accumulate in *sec*11 at 37°C, internal invertase levels rise 7 fold, and about one third of the accumulated invertase is secreted when cells are returned to 25°C. The *sec*11 gene product may be difficult to convert to a completely thermosensitive form, or it may not be required absolutely for the secretory process.

More important than the occasional leaky strain is the fact that in most *sec* mutants the block to secretion and membrane permease incorporation is coupled with the accumulation of secretory enzymes and membrane-enclosed organelles. van Rijn, Linnemans and Boer (1975) have shown by histochemical staining of wild-type cells that acid phosphatase is contained within ER, Golgi-like structures and vesicles. The acid phosphatase that accumulates in *sec*1 cells at 37°C is contained within vesicles (Novick and Schekman, 1979); the ER and Berkeley bodies (Bbs) produced in other mutants also contain this enzyme (B. Esmon, P. Novick and R. Schekman, manuscript in preparation). The various membrane-enclosed structures produced at 37°C probably represent functional intermediates in the secretory pathway, since most of the mutants secrete the accumulated invertase upon return to 25°C.

The rates of invertase synthesis and export may also be coupled. In many of the mutants, 2–4 fold more invertase accumulates in an internal pool at 37°C than is secreted by wild-type cells in a comparable period (Table 3).

Some of the mutants accumulate more than one

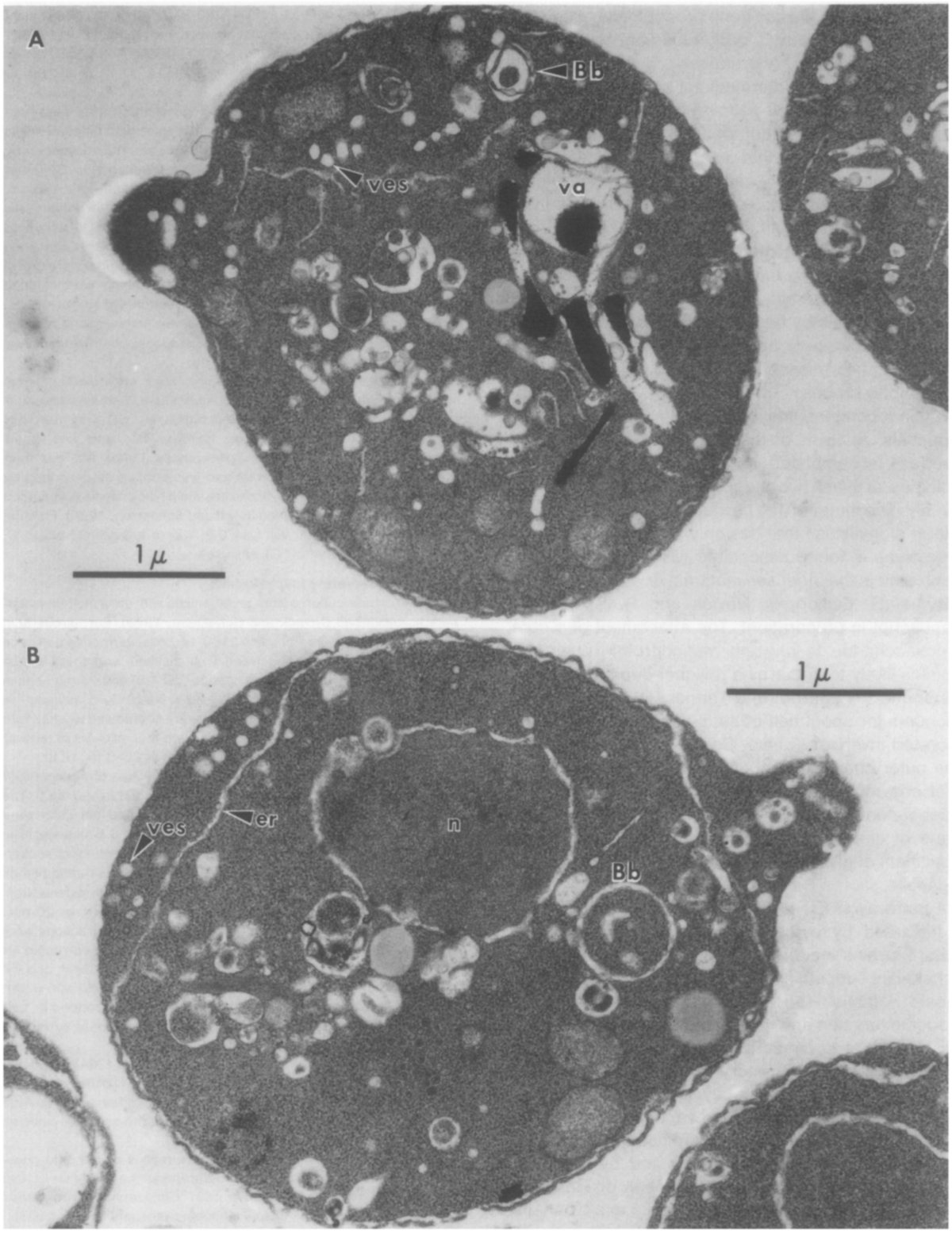

Figure 5. Thin Section Electron Micrographs of Cells Grown in YPD Medium at 25°C, Then Shifted to 37°C for 2 Hr
(A) HMSF 169 (*sec*14-3); (B) HMSF 178 (*sec*19-1). Symbols are as in Figures 2–4.

type of organelle. If a *sec* gene product acts at several stages in the pathway, a partial block might result in multiple structures. Alternatively, if the reactions which connect one intermediate to another are reversible, a block in the pathway could lead by mass action to the accumulation of an earlier intermediate. A third possibility is that unstable organelles may reversibly or irreversibly generate other structures.

The close resemblance of the structure in some *sec* mutants to ER suggests that the affected gene products are required at an early step in the pathway. The vesicle-accumulating mutants are probably defective in later steps. Bbs may be analogous to the Golgi apparatus; they may become distorted by the continued incorporation of new membrane and secretory material in the absence of discharge to a succeeding stage in the process.

An independent line of evidence supports these proposals. Analysis of the extent of glycosylation of invertase accumulated at 37°C in the *sec* strains indicates at least two stages in oligosaccharide assembly. The mutants that produce ER also accumulate a form of invertase that has only half as much carbohydrate as is found associated with the enzyme accumulated in the other *sec* mutants, or on the secreted enzyme (B. Esmon, P. Novick and R. Schekman, manuscript in preparation). The attachment of a core oligosaccharide to nascent mannoprotein chains in yeast is likely to occur by a dolichol-dependent reaction in the ER (Marriot and Tanner, 1979). This core accounts for about half of the carbohydrate found on secreted invertase (Lehle, Cohen and Ballou, 1979). The outer chain oligosaccharides are added by distinct enzymes (Raschke et al., 1973) in a dolichol-independent reaction (Parodi, 1979). This second stage of oligosaccharide assembly may occur after movement of glycoproteins from the ER to a Golgi-like organelle.

A pathway of ER → Bbs → vesicles → cell surface is indicated by cytological analysis of double *sec* mutant strains incubated at 37°C (P. Novick and R. Schekman, unpublished results). Further analysis should establish the order in which the *sec* gene products function and the relationship of this pathway to secretory and membrane protein maturation. The contribution of the secretory pathway to cell surface growth will be tested directly by the isolation and analysis of organelle and plasma membrane fractions from the *sec* mutant cells. By providing an enriched supply of intermediate organelles and by providing criteria for the authentic reconstruction of individual events in vitro, the *sec* mutants may aid in a biochemical dissection of the secretory pathway.

Experimental Procedures

Materials

S. cerevisiae isogenic haploid strains X2180-1A (*a*, gal2) and −1B (*α*, gal2) were from the yeast genetics stock center (Berkeley). NF1R, a spontaneous GAL$^+$ revertant, was derived from X2180-1A. HMSF 1 (*a*, *sec*1-1) was derived from X2180-1A (Novick and Schekman, 1979). Standard genetic techniques were used to construct SF182-3B (*a*, GAL$^+$) and SF150-5C (*a*, *sec*1-1, III *ACP*1-2, *pho*80-2). All other *sec* strains were derived from X2180-1A, NF1R or SF182-3B as described in the text.

YPD medium contained 1% Bacto-Yeast Extract, 2% Bacto-Peptone and 2% glucose; YP medium was the same with different levels of glucose. Phosphate-depleted YPD was prepared as described by Rubin (1973). Wickerham's minimal medium (Wickerham, 1946) was used with the following modifications: for phosphate-free medium, potassium chloride replaced potassium phosphate; for sulfate-free medium, chloride salts replaced all sulfate salts. Unless otherwise indicated, the carbon source was 2% glucose. Petri plates contained the indicated medium and 2% Difco agar. Liquid cultures were grown in flasks or tubes with agitation, and the experiments were initiated with exponentially growing cells from stock cultures at an A_{600} of 0.5–5. The absorbance of cell suspensions was measured in a 1 cm quartz cuvette at 600 nm in a Zeiss PM QII spectrophotometer; 1 A_{600} unit corresponds to 0.15 mg dry weight.

Other reagents were obtained as indicated: ethyl methanesulfonate, p-nitrophenolphosphate, glucose oxidase, O-dianisidine, peroxidase and cycloheximide were from Sigma; $H_2{}^{35}SO_4$ was from New England Nuclear; glutaraldehyde, osmium tetroxide and Spurr embedding medium were from Polysciences; Ludox AM was from Protex Wax (Oakland, California) and was purified as described by Price and Dowling (1977). Lyticase is a yeast lytic enzyme preparation useful in spheroplast formation (Scott and Schekman, 1980). Fraction II (30,000 U/mg; 1 unit will lyse 0.2 A_{600} of logarithmic phase S. cerevisiae in 30 min at 30°C) was used.

Isolation of Secretory (*sec*) Mutants

Stationary phase cultures were mutagenized with ethyl methanesulfonate as described (Novick and Schekman, 1979). For nitrous acid mutagenesis, stationary phase X2180-1A cells were collected on a nitrocellulose filter, washed twice with distilled water and resuspended in 5 ml of 0.5 M sodium acetate (pH 4.8) and 20 mg sodium nitrate. After 10 min at 30°C, 5 ml of 2.7% Na_2HPO_4 containing 1% yeast extract were added, and the cells were filtered and washed with water. The viability was 37%. The cells were then allowed to recover from mutagenesis by growth in YPD medium at 25°C for 16 hr.

In the largest enrichment experiment, 1.85 A_{600} of mutagenized cells were grown in 50 ml of YPD medium to a total A_{600} = 25.5. The culture was then incubated at 37°C for 3 hr and the cells were collected by filtration, washed and resuspended in 0.5 ml water. The cell sample was layered on 12.5 ml of a mixture containing Wickerham's salts (Wickerham, 1946) and 60% (v/v) of the purified stock Ludox suspension, in a Falcon 17 × 100 mm polypropylene tube. After centrifugation in a Sorval SS34 rotor (22,000 × g, 20 min, 4°C), the tube was punctured at the bottom and 1 ml fractions were collected. The A_{600} of the fractions was measured and corrected for the A_{600} of corresponding fractions from a cell-free gradient, and the densest 2% of the cells (1.5 A_{600} total) was diluted 2 fold with water. The cells were then centrifuged, the pellet was resuspended in 1 ml of water and diluted 400 fold, and 0.1 ml portions were spread on 200 YPD medium agar plates.

The plates were incubated at room temperature (20–25°C) for 2 days and colonies were replica-plated onto two YPD plates each; one replica was incubated at room temperature, the other at 37°C. After 26 hr the replicas were compared and temperature-sensitive colonies were picked from the master plate.

The temperature-sensitive clones were replica-plated onto phosphate-free minimal medium plates to derepress the synthesis of acid phosphatase, and after 10 hr at 24° or 37°C the replicas were stained for secreted acid phosphatase (Hansche, Beres and Lange, 1978).

The clones which showed temperature-sensitive secretion of phosphatase were screened for conditional secretion and internal accumulation of invertase. Cultures grown at 25°C in YP + 5% glucose medium were shifted to 37°C for 30 min, after which 2 A_{600} units of cells were sedimented for 1.5 min in a clinical centrifuge. The cell pellets were then resuspended in 2 ml of fresh YP + 0.1% glucose medium and cultures were incubated at 37°C for an additional 90 min. The cells were sedimented again and the pellets were resus-

pended in 10 mM NaN_3 at 0°C. Cell wall and internal invertase levels were measured as previously described (Novick and Schekman, 1979). Strains which secreted less invertase than the X2180 control were designated *sec* mutants; those that accumulated internal levels higher than the X2180 control were assigned to class A, and all others were put aside for further analysis.

Analytical Procedures

Cell number was determined with a hemocytometer; buds were counted as cells. Density was determined by sedimentation of 8 A_{600} units of cells on a Ludox gradient as described above. The region of highest cell concentration was estimated visually and a 0.5 ml sample was removed by puncturing the centrifuge tube with a syringe. Sample density was measured by weighing a 100 μl portion.

External (cell wall-bound) acid phosphatase was assayed at 37°C as described by van Rijn, Boer and Steyn-Parvé (1972); units of activity are nmole of p-nitrophenol released per min. External invertase was assayed at 37°C as described by Goldstein and Lampen (1975); units of activity are μmole of glucose released per min. Internal invertase was determined by assaying spheroplast lysates, prepared as previously described (Novick and Schekman, 1979). Sulfate permease was assayed at 37°C in 50 μM $(NH_4)_2SO_4$ as described by Breton and Surdin-Kerjan (1977); units of activity are nmole of SO_4^{2-} uptake per min. Radioactivity was measured in a Searle Delta 300 liquid scintillation counter.

Samples were prepared for electron microscopy by the procedure of Byers and Goetsch (1975).

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