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# Coincident Localization of Secretory and Plasma Membrane Proteins in Organelles of the Yeast Secretory Pathway

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**Immunoelectron microscopy of *Saccharomyces cerevisiae* cells embedded in Lowicryl K4M has been used to localize invertase and plasma membrane (PM) ATPase in secretory organelles. *sec* mutant cells incubated at 37°C were prepared for electron microscopy, and thin sections were incubated with polyclonal antibodies, followed by decoration with protein A-gold. Specific labeling of invertase was seen in the lumen of the endoplasmic reticulum, Golgi apparatus, and secretory vesicles in mutant cells that exaggerate these organelles. PM ATPase accumulated within the same organelles. Double-immune labeling revealed that invertase and PM ATPase colocalized in secretory vesicles. These results strengthen the view that secretion and plasma membrane assembly are biosynthetically coupled in yeast.**

The secretory pathway has been defined in *Saccharomyces cerevisiae* by the isolation and characterization of a series of conditionally lethal mutations in which protein transport is arrested intracellularly (15). Biochemical and cytological inspection revealed secretory proteins accumulated in the endoplasmic reticulum, Golgi apparatus, or secretory vesicles, depending on the *SEC* gene that was affected (7, 16). Analysis of cell surface membrane proteins suggested that plasma membrane (PM) components also are transported to the surface via the secretory pathway (17, 24). Unfortunately, techniques for the visual localization of PM precursor proteins in transit to the cell surface were not developed.

To confirm our prediction that secretory and PM precursor proteins are transported within the same organelles, we developed an immunoelectron microscopic approach to detect antigens on thin sections of yeast cells. Antibodies directed against invertase, a secreted glycoprotein, and PM ATPase, an integral membrane protein, decorated the same organelles in various strains defective in secretion.

## MATERIALS AND METHODS

**Strains, growth conditions, and materials.** *S. cerevisiae* X2180-1A (wild type), HMSF176 (*sec18*), SF294 (*sec7*), *SF263* (*sec1*), SEY5188 (*sec18 suc2-Δ9 leu2-3 leuα-112 ura3-52*), SEY5078 (*sec7 suc2-Δ9 leu2-3 leu2-112 ura3-52*), and SEY5018 (*sec1 suc2-Δ9 leu2-3 leu2-112 ura3-52*) were described previously (6, 15). *Escherichia coli NK5772 (dcm-6 dam-3 galK2 galT22 metB1 levY1 tsx-78 thi-1 tonA31 mtl-1)* was from J. Kadanaga (this department). A 2μm plasmid derived from YEp24, pRB58, contains the *SUC2* gene (4). Standard techniques were used to introduce plasmids pRB58, YEp24, and pDB31 into yeast strains SEY5188, SEY5078, and SEY5018.

YPD medium contained 1% yeast extract (Difco Laboratories, Detroit, Mich.), 2% Bacto-Peptone (Difco), and 2 or 5% glucose. Wickerham minimal medium (26) was used with glucose as a carbon source. The $A_{600}$ of the cell suspension was measured in a Zeiss PMA II spectrophotometer.

$^{125}$I-labeled Na was from Amersham Corp., Arlington Heights, Ill. Nitrocellulose was from Schleicher & Schuell, Inc., Keene, N.H. Restriction enzymes were purchased from Bethesda Research Laboratories, Gaithersburg, Md. CNBr-activated Sepharose 4B and protein A were obtained from Pharmacia Fine Chemicals, Piscataway, N.J. Tetrachloroauric acid and polyethylene glycol (molecular weight, 20,000) were obtained from E. Merck AG, Darmstadt, Federal Republic of Germany. Lowicryl K4M, Lowicryl HM20, and RL gold were obtained from Polysciences, Inc., Warrington, Pa. Protein A was iodinated by the chloramine T procedure (9).

**Preparation of immunoreagents.** Production of antibodies against invertase was reported previously (20, 22). ATPase was purified according to the method of Malpartido and Serrano (13). Antiserum against the sodium dodecyl sulfate-denatured subunit of ATPase was a gift from Nathan Nelson (Roche Institute of Molecular Biology, Nutley, N.J.). Protein A-gold complexes were prepared as reported elsewhere (19, 21).

All polyclonal antisera used in this study were treated to remove contaminating cell wall carbohydrate antibodies. $NaN_3$ was added to X2180-1A cells (which had an optical density at 600 nm [$OD_{600}$] of 50) to a final concentration of 10 mM at 4°C. The cells were then washed twice with phosphate-buffered saline (PBS) containing 1 M NaCl–0.01% $NaN_3$. Antiserum (1 ml) was incubated with the washed cells at 4°C overnight. The cells were removed by centrifugation, and the serum was treated again with fresh cells. Mannan (75 mg), prepared from commercial yeast (Red Star Yeast, Oakland, Calif.) by precipitation with Fehling reagent (10), was coupled to CNBr-activated Sepharose 4B (1.25 g) according to instructions provided by Pharmacia. The conjugate was washed in PBS plus 10 mm $NaN_3$, and 2 ml was mixed with adsorbed serum to remove final traces of carbohydrate antibody. For adsorption of invertase antiserum, mannan was prepared from a *suc2* deletion mutant strain.

**Construction of plasmid pDB31.** pDB31 contained the triosephosphate isomerase (TPI) promoter adjacent to the *SUC2* gene, allowing glucose-unregulated expression of invertase (see Fig. 5). Plasmid pCG70 (obtained from M. Duncan, Collaborative Research, Lexington, Mass.) contained the TPI promoter adjacent to the 5′ coding segment of *SUC2* up to the first *Hin*dIII site. An approximately 2-kilobase *Hin*dIII fragment from YCp50 (4) containing the

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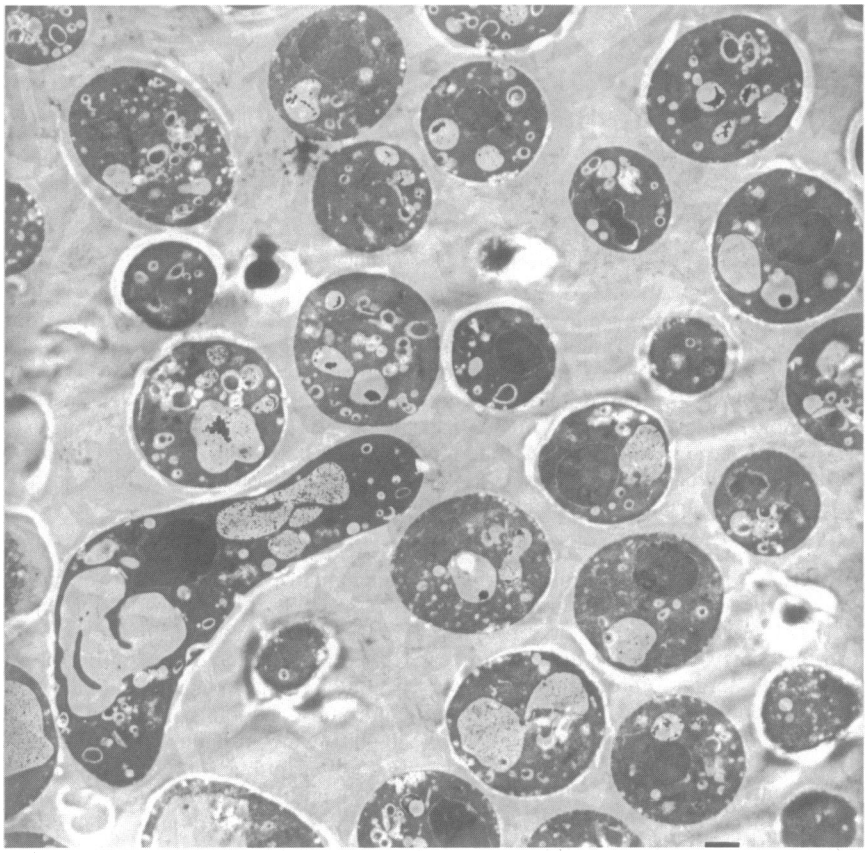
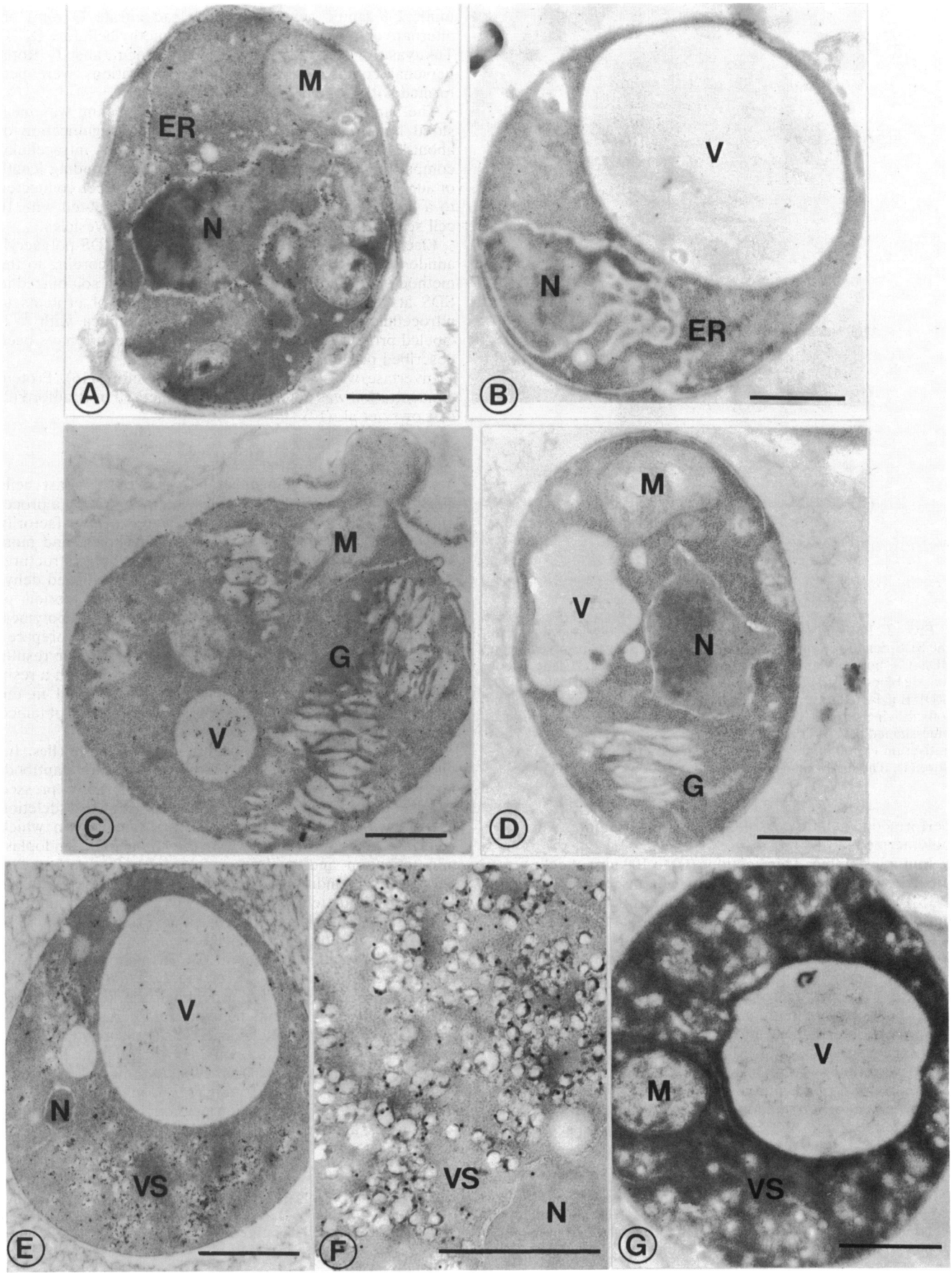


FIG. 1. Thin section of yeast cells embedded in Lowicryl K4M. *sec7* mutant cells were incubated at 37°C for 1.5 h and prepared for electron microscopy. Bar, 1 μm.

remaining coding sequence of *SUC2* was purified and ligated to a *Hin*dIII nuclease-restricted linear form of pCGS670. This plasmid contained only a partial *URA3* gene. To facilitate selection in yeast, we exchanged the *Bcl*I-*Bam*HI fragment in pRB58 (4), which contained a part of the *SUC2* gene and some upstream sequences, for the *Bgl*II-*Bam*HI fragment from the construct described above, which contained the TPI promoter and part of the invertase gene. For this purpose, pRB58 (propagated in *E. coli* NK5772) was subjected to partial digestion with *Bcl*I nuclease and the linearized plasmid was treated with *Bam*HI nuclease. Finally, the *Bgl*II-*Bam*HI fragment was ligated into the linearized pRB58.

**Low-temperature embedding of yeast in Lowicryl K4M.** Untransformed yeast cells were grown in YPD medium to an $OD_{600}$ of 0.5 to 2. Cells containing plasmids were first grown on minimal selective medium to an $OD_{600}$ of 1 to 4, diluted in YPD to an $OD_{600}$ of 0.5, and grown for 5 to 6 h to an $OD_{600}$ of 1. Cells (50 $OD_{600}$ U) were washed twice with 10 ml of yeast extract–Bacto-Peptone and 0.1% glucose, resuspended in 25 ml of the same medium, and incubated for 1.5 h at 37°C. Cells in YPD medium were shifted to 37°C directly. $NaN_3$ was added to a final concentration of 10 mM in cultures that were cooled on ice. Cells (50 $OD_{600}$ U) were then washed twice with 5 ml of 0.1 M cacodylate (pH 6.8)–1.5 mM $CaCl_2$–2.5 mM $MgCl_2$. Initial mild fixation was performed for 30 min at 20°C with 2% formaldehyde–0.1% glutaraldehyde in 0.1 M cacodylate buffer (pH 6.8)–1.5 mM $CaCl_2$–2.5 mM $MgCl_2$. Cell walls were removed with lyticase (15), followed by further fixation at 20°C for 30 min or at 4°C for 12 h. Fixed cells were embedded in 2% low-melting-point agarose (Bethesda Research Laboratories) and cut into small pieces. Low-temperature embedding was performed by a modification of the published method (3). Samples were dehydrated in graded ethanols: 50% ethanol at 0°C for 1 h, 70% at −20°C for 1 h, 90% at −35°C for 1 h, and 100% at −35°C for 24 h (seven changes). Dehydrated samples were infiltrated with a mixture of ethanol and Lowicryl K4M (1:1) at −35°C for 1 h and with prepolymerized resin at −35°C for a total of 50 h (three changes). Lowicryl K4M was prepolymerized at −35°C for 30 min. Samples were mixed by hand occasionally during dehydration and infiltration. Final embedding was

FIG. 2. Invertase accumulated within secretory organelles. Cells were grown in yeast extract–Bacto-Peptone–2% glucose at 24°C and transferred to yeast extract–Bacto-Peptone–0.1% glucose for 90 min at 37°C to deregress invertase synthesis. Samples prepared for electron microscopy were sectioned and stained with invertase antiserum or affinity-purified antibodies, followed by protein A-gold. (A, C, E, and F) *sec* mutant strain transformed with the multicopy *SUC2* plasmid pRB58. (B, D, and G) *sec* mutant cells containing a *suc2* deletion and transformed with a control plasmid, YEp24. (A and B) *sec18*; (C and D) *sec7*; (E, F, and G) *sec1*. Bars, 1 μm. Abbreviations: N, nucleus; ER, endoplasmic reticulum, V, vacuole; G, Golgi apparatus; M, mitochondrion; VS, vesicles.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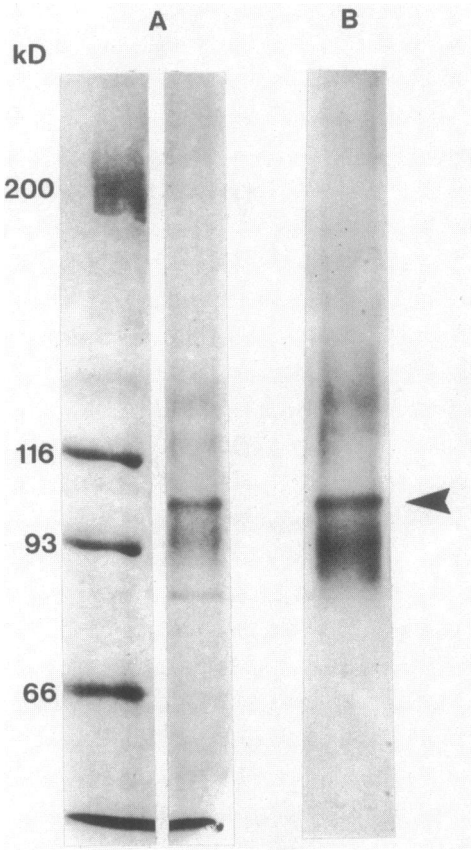


FIG. 3. PM ATPase is recognized by an antibody raised against the SDS-denatured subunit. Purified PM ATPase was resolved on an SDS–6% polyacrylamide electrophoretic gel. (A) Left lane, Coomassie blue-stained molecular mass marker (myosin, 200 kilodaltons [kD]; β-galactosidase, 116 kilodaltons; phosphorylase *b*, 93 kilodaltons; bovine serum albumin, 66 kilodaltons). Right lane, Coomassie blue-stained PM ATPase. (B) PM ATPase reacted with ATPase antiserum (1:500 dilution) and decorated by the golden blot procedure (1). The arrowhead indicates the position of PM ATPase.

performed in gelatin capsules in which the samples were UV polymerized.

**Immunostaining.** Thin sections (60 to 100 nm) of cells were incubated at 24°C on a drop of 0.1% bovine serum albumin in PBS (with 0.5 M NaCl; pH 7.5) for 5 min and then with antibodies in the same solution for 2 h at 24°C or for 12 h at 4°C. Protein A-gold was diluted in PBS (pH 7.4)–1% Triton X-100 to an $A_{525}$ of 0.13 (for a particle size of 8 nm) or 0.3 (for a particle size of 14 nm). After a wash in PBS, grids were placed on a drop of protein A-gold complex for 45 min at 24°C. For a second immune labeling, grids were treated with 0.1 mg of protein A per ml for 5 min, washed, and exposed to the next serum, as described above. Washing and exposure to protein A-gold (of a different size) were also conducted as described above. Finally, grids were washed in PBS–1% Triton X-100, and in PBS, and in distilled water and then dried. Sections were stained with 2% uranyl acetate (25 min), 2% tannic acid (5 s), and lead citrate (3 min) or, alternatively, with uranyl acetate and methylcellulose (K. T. Tokuyasu, D. J. Taatijes, J. M. Lucocq, and J. Roth, personal communication). Control incubations were performed with protein A-gold and no antibody.

The surface area and density of gold labeling was measured by projecting negatives to a final magnification of about 125,000-fold. Gold particles over the intracellular compartments were counted, and the corresponding length or surface area was determined by using a tracer connected to a computer. Each measurement was conducted with 10 cell sections and included approximately 500 vesicles.

**Electrophoretic procedures and assays.** SDS-polyacrylamide gel electrophoresis was carried out according to the method of Laemmli (11) on samples that were solubilized in SDS at 50°C (8a). Electrophoretic transfer of proteins to nitrocellulose, treatment of the immunoreplica with $^{125}$I-labeled protein A, and the golden blot procedure have been described previously (1).

Invertase was assayed as described before (16). Protein concentration was determined by the method of Bradford (2) or Lowry et al. (12).

## RESULTS

**Low-temperature embedding of yeast cells.** Yeast cells embedded in Lowicryl K4M at low temperature by a procedure used for mammalian cells (3) were not satisfactorily preserved. Thin sections contained many holes, and most cells were dark, with little apparent intracellular structure. Satisfactory preservation was obtained by prolonged dehydration in absolute ethanol and an extended period of infiltration with resin combined with the use of prepolymerized Lowicryl K4M. An example of a thin section prepared by these modifications is shown in Fig. 1. Similar results were obtained when embedding was performed with a resin mixture that had less cross-linker than was used in the sample infiltration. Comparable results also were obtained with Lowicryl HM20 and RL gold resins.

**Accumulation of invertase within secretory organelles.** Invertase antiserum, purified to remove carbohydrate antibodies, was used to label thin sections of cells that expressed and accumulated invertase and thin sections of *suc2* deletion mutant cells. Figure 2A shows a *sec18* mutant in which invertase was accumulated within the lumen of the endoplasmic reticulum and nuclear envelope. *sec7* mutant cells, which under conditions of derepressed invertase synthesis (0.1%) accumulate Golgi apparatus (14), showed uniform labeling of the multiple cisternae that proliferated at 37°C (Fig. 2C). Secretory vesicles that accumulated when *sec1* mutant cells were incubated at 37°C also showed extensive immune reaction with the invertase antibody (Fig. 2E and F). Staining seemed to be enriched in darker, pocketlike inclusions on the luminal face of the secretory vesicles. Immune labeling was enhanced in cells that carried *SUC2* on a multicopy plasmid, although, as with normal levels of enzyme, the pattern varied somewhat among cells. Control incubations with *suc2* deletion, *sec* double mutant cells showed organelle proliferation without invertase staining (Fig. 2B, D, and G).

FIG. 4. PM ATPase in secretory organelles. *sec* mutant strains were incubated at 37°C for 90 min and prepared for electron microscopy. (A, C, and E) Thin sections were treated with ATPase antiserum, followed by protein A-gold. (B, D, and F) Specificity of labeling was tested by preincubation of the antiserum with purified native ATPase (39 μg for 20 μl of serum) or SDS-denatured ATPase (1 μg for 20 μl of serum). (A and B) *sec18*; (C and D) *sec7*; (E and F) *sec1*. Bars, 1 μm. Abbreviations are defined in the legend to Fig. 2.

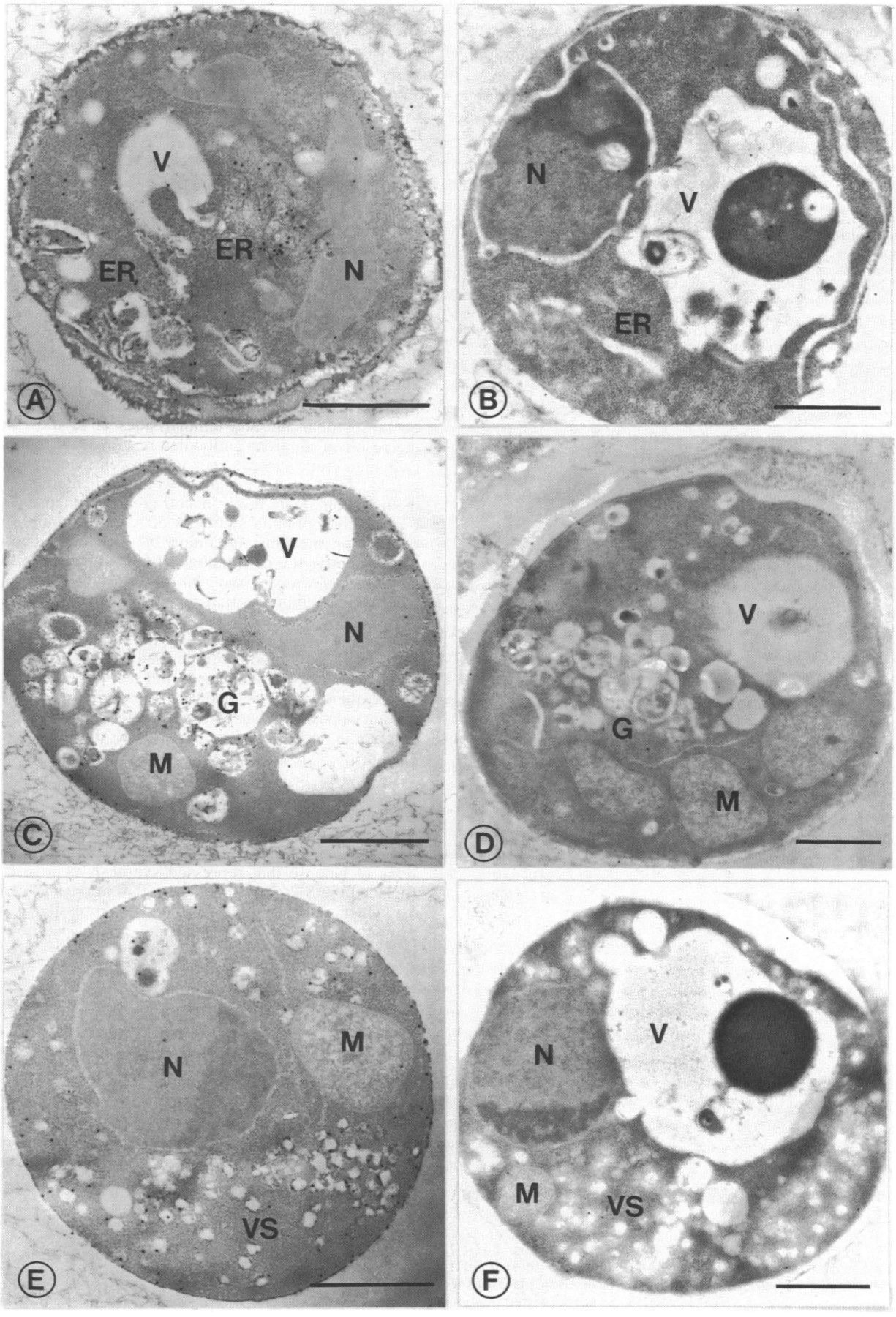
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TABLE 1. Labeling density of gold particles on sections of *sec1* cells labeled with ATPase antibody

| Organelle(s) | No. of particles with[a]: | |
|---|---|---|
| | Purified antiserum | Control |
| Small vesicles | 60.5 ± 4.2 | 2.6 ± 1.2 |
| Nucleus | 0.5 ± 0.3 | 0.2 ± 0.2 |
| Vacuole | 4.0 ± 3.5 | 0.6 ± 0.7 |
| Mitochondria | 0.5 ± 0.6 | 1.0 ± 1.2 |
| Cytoplasm | 2.2 ± 0.9 | 1.2 ± 0.7 |
| Plasma membrane | 2.2 ± 0.1 | 0.2 ± 0.2 |
| Endoplasmic reticulum | 0.1 ± 0.1 | 0.1 ± 0.1 |

[a] In the small vesicles, nucleus, vacuole, mitochondria, and cytoplasm, the number of particles per square micrometer were counted. In the plasma membrane and endoplasmic reticulum, the number of particles per micrometer were counted. The control was antiserum preadsorbed with antigen. Values are expressed as means ± the standard deviations.

**PM ATPase in secretory organelles.** Polyclonal antiserum raised against purified PM ATPase recognized the protein on an immunoblot (Fig. 3) and specifically precipitated the correct protein from detergent-solubilized membranes (8a). Immunolabeling of thin sections was performed with or without preincubation of antiserum with excess purified PM ATPase (Fig. 4). ATPase-specific labeling was detected

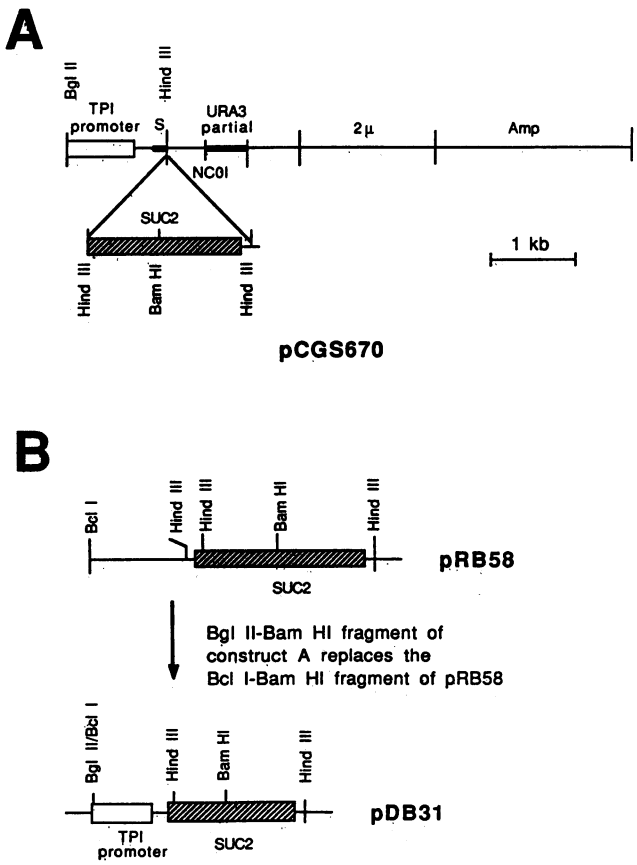


FIG. 5. Construction of pDB31. (A) A *Hin*dIII fragment of *SUC2* was inserted at the *Hin*dIII site of pCGS670. (B) A *Bgl*II-*Bam*HI fragment of the construct in panel A was inserted in place of the *Bcl*I-*Bam*HI fragment of *SUC2* in pRB58. S, Partial signal peptide contained at the *Hin*dIII site in pCGS670.

TABLE 2. Constitutive secretion of invertase promoted by pBD31

| Plasmid[a] | % Glucose in medium | Invertase activity[b] | | | |
|---|---|---|---|---|---|
| | | Secreted | | Intracellular | |
| | | U | % | U | % |
| pRB58 | 5 | 278 | 87 | 40 | 13 |
| | 0.1 | 1,081 | 96 | 47 | 4 |
| pDB31 | 5 | 1,207 | 96 | 45 | 4 |
| | 0.1 | 1,226 | 93 | 93 | 7 |

[a] X2180-1A cells harboring pRB58 or pDB31 were grown in yeast extract–Bacto-Peptone medium containing 5 or 0.1% glucose for 90 min at 37°C. Cells were collected by centrifugation and converted to spheroplasts to separate secreted and intracellular (spheroplast) invertase activity.
[b] One unit of invertase produces 1 nmol of glucose per min.

along profiles of intracellular tubules and the plasma membrane in *sec18* cells (Fig. 4A). Gold particles appeared both on the cytoplasmic and luminal sides of tubules, possibly the result of different antibodies reacting with epitopes on each membrane surface. *sec7* mutant cells accumulate exaggerated Golgi cisternae only when incubated at 37°C in medium containing low concentrations of glucose (14). Unfortunately, optimum synthesis of ATPase required growth of cells on medium containing 2% or more glucose (8a); consequently, the morphology of Golgi structures accumulated in *sec7* was aberrant. Nevertheless, ATPase antibody labeled the membranes that accumulated in this mutant (Fig. 4C). Finally, secretory vesicle membranes, accumulated in *sec1* cells, were also labeled by the antibody (Fig. 4E). In each case, competition with purified ATPase reduced significantly the antibody labeling of thin sections (Fig. 4B, D, and F). A quantitative treatment of the compartments in *sec1* mutant cells showed a significant enrichment of gold-labeled ATPase in small vesicles compared to that in other organelles or the cytoplasm (Table 1).

**Coincident localization of invertase and PM ATPase.** In order to confirm the coincidence of ATPase and invertase in compartments of the secretory pathway, it was necessary to develop a condition in which both proteins were expressed simultaneously. Since ATPase synthesis required concentrations of glucose that repressed synthesis of invertase, we replaced the *SUC2* promoter with that of the TPI gene (Fig. 5), which is insensitive to glucose repression. The TPI promoter-*SUC2* fusion on a plasmid (pDB31) was introduced into *suc2* chromosomal deletion mutant strains. A compari-

TABLE 3. Labeling density of gold particles on sections of *sec1* cells doubly labeled for ATPase and invertase

| Organelle(s) | No. of particles with labeling for[a]: | |
|---|---|---|
| | ATPase | Invertase |
| Small vesicles | 70.5 ± 21.2 | 91.1 ± 26.7 |
| Nucleus | 0.3 ± 0.2 | 0.3 ± 0.2 |
| Vacuole | 2.3 ± 1.1 | 3.3 ± 0.8 |
| Mitochondria | 0.4 ± 0.5 | 0.2 ± 0.3 |
| Cytoplasm | 3.0 ± 1.3 | 3.9 ± 1.2 |
| Plasma membrane | 2.4 ± 1.0 | 0.1 ± 0.1 |
| Endoplasmic reticulum | 0.2 ± 0.2 | 0.1 ± 0.1 |

[a] In the small vesicles, nucleus, vacuole, mitochondria, and cytoplasm, the number of particles per square micrometer were counted. In the plasma membrane and endoplasmic reticulum, the number of particles per micrometer were counted. Organelles labeled for ATPase were marked by 14-nm particles; those labeled for invertase were marked by 8-nm particles. Values are expressed as means ± the standard deviations.

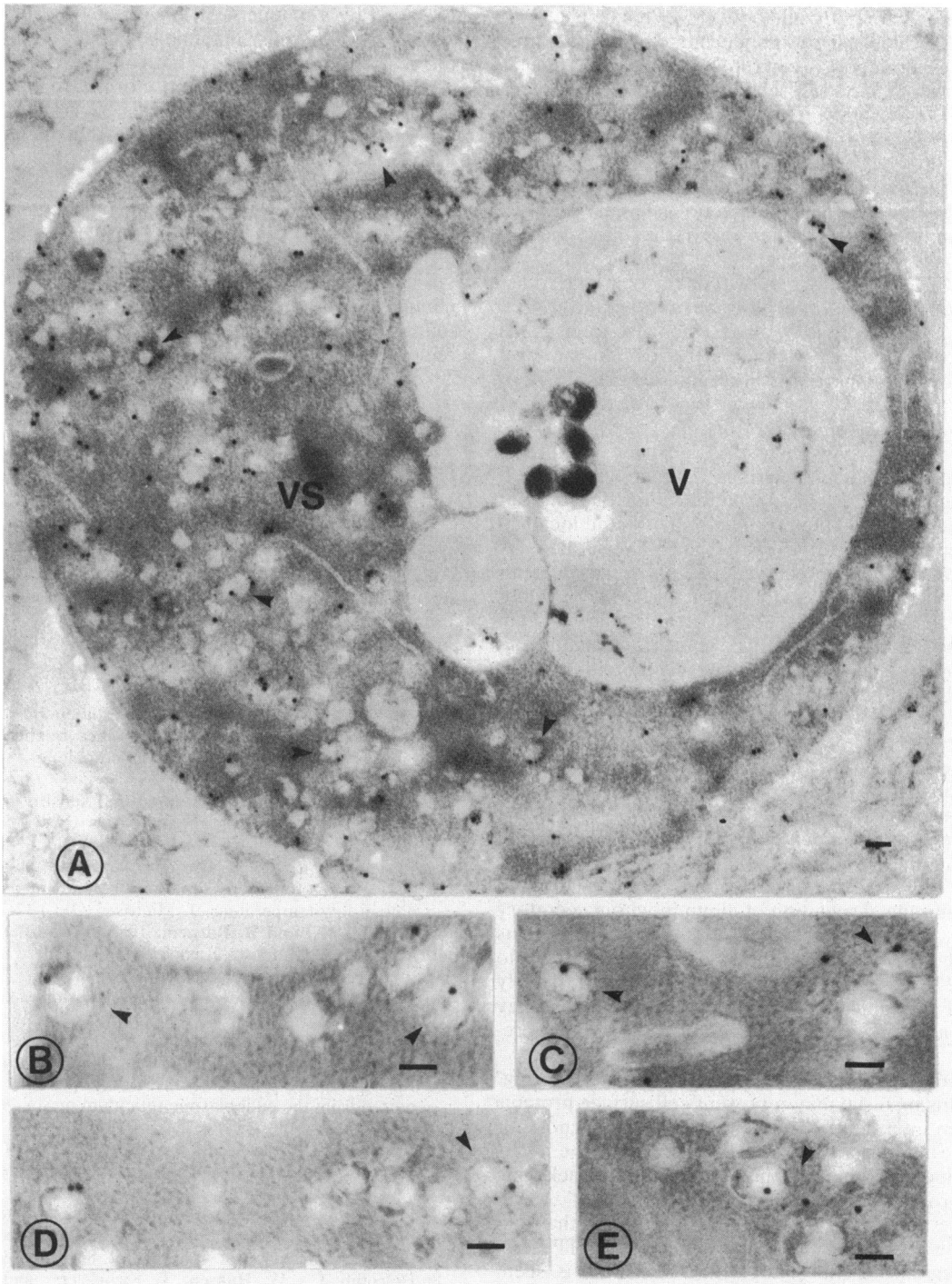


FIG. 6. Coincident immunolabeling of ATPase and invertase in secretory vesicles. *sec1* cells transformed with pDB31 were incubated at 37°C for 90 min and prepared for electron microscopy. Thin sections were treated subsequently with invertase antiserum followed by protein A-gold (8 nm) and ATPase antiserum followed by protein A-gold (14 nm). Bars, 0.1 μm. Abbreviations are defined in the legend to Fig. 2. Arrowheads indicate vesicles that contain invertase and PM ATPase.

son of invertase secretion at low and high glucose levels in cells that contained the normal *SUC2* gene (plasmid pRB58) or the fusion gene (pDB31) confirmed glucose insensitivity of the latter (Table 2).

Thin sections of *sec1* mutant cells containing pDB31 were doubly stained with anti-invertase and anti-PM ATPase conjugates; those stained with anti-invertase were marked by small (8-nm) gold particles, and those stained with anti-PM ATPase were marked by large (14-nm) particles. Figure 6 shows that many of the secretory vesicles that accumulated at 37°C were tagged by particles of both sizes. Quantitation of the vesicles in 10 cells (about 500 vesicles total) showed that 61% of the vesicles were labeled with small gold particles, 67% were labeled with large particles,

46% were labeled with both, and only 18% were labeled with neither particle. Table 3 shows the density of small and large gold conjugates enriched in secretory vesicles relative to other membranes in *sec1* cell sections.

## DISCUSSION

We have developed and applied an immunoelectron microscopic technique that allows detection of yeast intracellular protein antigens. Two other approaches have yielded compelling images of carboxypeptidase Y in the yeast vacuole (25) and HMG-coenzyme A reductase in the yeast nuclear envelope (R. Wright, M. Basson, and J. Rine, J. Cell Biol., in press). Our approach was designed also to visualize organelles of the secretory apparatus that accumulate at a restrictive growth temperature in yeast secretory mutant cells.

Previous work from our laboratory has shown that the secreted protein acid phosphatase is localized within the organelles that accumulate in various *sec* mutant strains (7, 16). These studies used a histochemical stain that did not resolve sufficiently to distinguish membrane-bound localization from luminal localization of the phosphatase activity. Other technical problems precluded acid phosphatase localization in the vacuole and in Golgi structures that accumulate in *sec7* mutant cells incubated at 37°C in medium containing low concentrations of glucose. Immunogold staining of invertase demonstrated that this protein accumulates uniformly within the endoplasmic reticulum and Golgi cisternae of *sec18* and *sec7* cells, respectively (Fig. 2A and C).

Secretory vesicles that accumulated in *sec1* cells were labeled with invertase antibody along the luminal surface of the membrane (Fig. 2F and 6A through E). Gold particles frequently appeared in association with regions of enhanced staining within the vesicles. Immunoelectron microscopic inspection of a liver cell line (HEp-2) has also shown an apparent membrane association of albumin within secretory vesicles (23). Although it is unlikely that secretory proteins engage specific membrane receptors during packaging into vesicles (18, 27), adsorption to a membrane surface, possibly in the form of a complex of diverse secretory proteins, may explain this biased location of soluble proteins within secretory vesicles.

Invertase staining also was detected in the vacuole (Fig. 2C and E). This material may represent cell surface proteins that have been internalized and delivered to the vacuole for degradation (5). Alternatively, a small fraction of the invertase may escape the normal sorting process during packaging of vacuolar proteins in the Golgi apparatus (22).

PM ATPase appears in the same compartments that mediate invertase secretion. No segregation of the ATPase in specialized regions of the endoplasmic reticulum is seen. Unfortunately, conditions that allow maximum synthesis of the ATPase (>2% glucose in the growth medium) are incompatible with proliferation of stacks of Golgi cisternae in *sec7* cells (8a). Instead, under conditions of optimal ATPase synthesis, degenerate and irreversible structures called Berkeley bodies accumulate at 37°C (14). Hence, although ATPase appears uniformly distributed among the membranes of Berkeley bodies, no firm conclusion can be drawn about possible differentiation among Golgi cisternae in regard to content of PM protein precursors.

Coincident localization of invertase and PM ATPase in a large fraction of the vesicles that accumulate in *sec1* cells confirms the hypothesis that secretion and bud growth are executed by a unique vesicle species (8). Since the level of ATPase and invertase immune labeling is quite low, not all of the vesicles are tagged with both antibodies. This appears to be a problem of antigen quantity or accessibility and not the result of multiple distinct vesicles. Corroborating cell fractionation experiments have shown that newly synthesized PM ATPase coincides exactly with acid phosphatase during successive steps in the purification of secretory vesicles from *sec1* cells (8a). A similar coincidence has been visualized in a liver cell line (HEp-2) in which the G membrane protein of vesicular stomatitis virus is immunolabeled in vesicles that also contain the soluble proteins transferrin and albumin (23). Hence, mechanisms exist in all eucaryotic cells to allow packaging of diverse membrane and secretory molecules into a common vesicle carrier.

## ACKNOWLEDGMENTS

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