Novel Peroxisome Clustering Mutants and Peroxisome Biogenesis Mutants of *Saccharomyces cerevisiae*

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Abstract. The goal of this research is to identify and characterize the protein machinery that functions in the intracellular translocation and assembly of peroxisomal proteins in *Saccharomyces cerevisiae*. Several genes encoding proteins that are essential for this process have been identified previously by Kunau and collaborators, but the mutant collection was incomplete. We have devised a positive selection procedure that identifies new mutants lacking peroxisomes or peroxisomal function. Immunofluorescence procedures for yeast were simplified so that these mutants could be rapidly and efficiently screened for those in which peroxisome biogenesis is impaired. With these tools, we have identified four complementation groups of peroxisome biogenesis mutants, and one group that appears to express reduced amounts of peroxisomal proteins. Two of our mutants lack recognizable peroxisomes, although they might contain peroxisomal membrane ghosts like those found in Zellweger syndrome. Two are selectively defective in packaging peroxisomal proteins and moreover show striking intracellular clustering of the peroxisomes. The distribution of mutants among complementation groups implies that the collection of peroxisome biogenesis mutants is still incomplete. With the procedures described, it should prove straightforward to isolate mutants from additional complementation groups.

PEROXISOME biogenesis occurs by the import of proteins posttranslationally, following synthesis on cytosolic free polyribosomes (19, 41). The membrane of the peroxisome grows by the incorporation of newly synthesized membrane proteins and, presumably, lipids. The peroxisome is capable of division by fission to form new peroxisomes, and segregation upon cell division, even when the membrane is nearly empty of the proteins that are normally packaged within it. This conclusion is based on studies of cells from human patients with Zellweger syndrome, in which peroxisomal membranes are present, but the import of proteins inside the peroxisomes is defective (27, 28).

Three types of targeting sequences have been discovered thus far, each of which functions to direct some proteins to peroxisomes. A noncleaved carboxy-terminal SKL tripeptide is sufficient to target proteins to peroxisomes in several species (10, 11), and is present on many peroxisomal proteins (12). Variants of the SKL tripeptide function in several yeasts (1). Rat thiolase contains an amino-terminal, cleavable peroxisome targeting oligopeptide (24, 38). *Candida tropicalis* acyl-CoA oxidase has two internal, redundant topogenic peptides (35). Catalase A of *Saccharomyces cerevisiae* has both a noncleaved COOH-terminal SSNSKF and an internal topogenic sequence (17).

To fully understand the import process, it is critical to identify and characterize the proteins that make up the machinery for the translocation of newly synthesized proteins into peroxisomes. Several such proteins have been identified recently (7, 15, 31, 47). This progress has been the result of genetic experiments in which mutant cells that were defective in peroxisome biogenesis were isolated. The genes were then cloned by functional complementation and sequenced. The mutants were made in CHO cells (40, 50) and in several species of yeast (4–6, 13, 20, 42).

Peroxisomes may be induced in *S. cerevisiae* by growth in the presence of fatty acids, such as oleate, and repressed by growth in glucose (45). Yeast peroxisomes can be isolated by cell fractionation (21, 39), and an in vitro import assay has been established to study the translocation of newly synthesized proteins into the organelle (39). These features make *S. cerevisiae* an attractive model organism for analysis of peroxisome biogenesis.

Some peroxisome assembly mutants in *S. cerevisiae* were identified by screening for cells unable to use oleic acid as the sole carbon source (6). Several of these mutants had a phenotype resembling Zellweger syndrome, but there were far fewer than the nine complementation groups that have been demonstrated thus far among human patients (30, 48). Therefore, we set out to devise a positive selection procedure to find additional mutants.

Strategy for Isolation of Peroxisome Biogenesis Mutants

In *S. cerevisiae*, fatty acid β-oxidation occurs in peroxi-

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somes, which proliferate when the yeast is grown in medium containing fatty acids (32, 45). H_2O_2 is a byproduct of this metabolism, one H_2O_2 being produced each time a fatty acid is shortened by two carbons. H_2O_2 is very toxic if it accumulates in the cell. It is mainly degraded by two isozymes of catalase: catalase A within the peroxisome and catalase T in the cytosol (Fig. 1). We hypothesized that if a yeast strain lacking both catalase A and catalase T were to be grown in a medium containing oleic acid, the H_2O_2 generated during the β-oxidation of oleic acid might accumulate in the cell and cause DNA damage or other cytotoxicity. This could result in the retardation of cell growth or even cell death.

We further supposed that if the yeast strain lacking catalase activity were mutagenized, any mutants in which peroxisome biogenesis failed might be unable to carry out peroxisomal fatty acid β-oxidation, and therefore would not produce H_2O_2 when grown in the presence of oleic acid. In the absence of H_2O_2 cytotoxicity, such mutants would be expected to grow normally, provided that an alternative carbon source were available. The normal-growing mutants could be readily identified and isolated for further analysis (Table I). This would be the basis for a positive selection procedure.

We tested this hypothesis, with the results described below. A preliminary account of this work has appeared (Zhang, J. W., Y. Han, and P. B. Lazarow. 1991. *J. Cell Biol.* 115:233a). Recently, after the completion of these experiments, a variant of this procedure has been described (42).

Materials and Methods

Yeast Strains and Media

The yeast strains used in this study are described in Table II. YPD, YPG, synthetic medium, synthetic minimal media with various nutrients, and the sporulation medium were prepared as described by Sherman et al. (29). YPOT was prepared according to Thieringer et al. (39). YPGO contains 0.1% (wt/vol) oleic acid and 0.25% (vol/vol) Tween 40 added to YPG medium. YNO was prepared according to Erdmann et al. (6). 2% (wt/vol) Bacto-agar was added to the appropriate media to make plates.

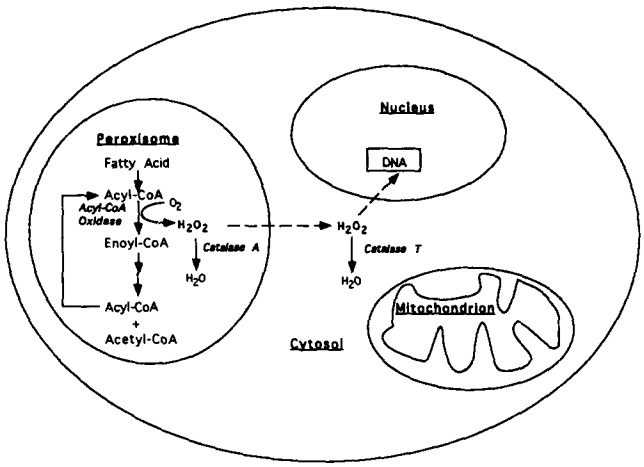


Figure 1. Protection against H_2O_2 toxicity in *S. cerevisiae*. H_2O_2 is formed in peroxisomes when a double bond is produced in a fatty acid undergoing β-oxidative conversion to acetyl-CoA. The H_2O_2 is normally decomposed within the peroxisome by catalase isozyme A. Any H_2O_2 escaping from the peroxisome will be degraded by the cytosolic catalase isozyme T before it can damage other macromolecules, such as DNA.

Table I. Strategy to Isolate Peroxisome Biogenesis (Peb) Mutants Based on the Toxicity of H_2O_2

	Yeast strains		
	Wild type	Catalase-deficient	*Peb* mutant of catalase-deficient
Growth medium: carbon source	Glycerol and oleate	Glycerol and oleate	Glycerol and oleate
Fatty acid oxidation and production of H_2O_2	+	+	–
Catalase activity and decomposition of H_2O_2	+	–	–
Accumulation of H_2O_2	–	+	–
Expected growth	Normal colonies	Tiny or no colonies	Normal colonies

Immunofluorescence

The immunofluorescence procedure of Pringle et al. (25) was simplified in order to analyze 50–100 clones daily. Cells were precultured in 2 ml of YPD overnight, inoculated into YPGO medium at a 1:50-dilution, and grown for 18 to 20 h. The cells were fixed by adding 1/10 volume of 37% formaldehyde to the culture. The fixation time was typically 1 h at room temperature although times ranging from 20 min to 2 h were satisfactory. The cells were then washed twice with 100 mM phosphate buffer (pH 7.4) and once with SP buffer (1.2 M sorbitol, 20 mM potassium phosphate buffer, pH 7.4). Cell walls were digested with Zymolyase (10 μg/ml) in SP buffer containing 1 μl/ml β-mercaptoethanol at 30°C for 30 min. In most cases, ~70% of the cells were converted to spheroplasts. The spheroplasts were washed twice with SP buffer and resuspended in 30 μl of SP buffer. 1 μl of cell suspension was applied to a multi-well slide coated with poly-L-lysine (each well can hold four to six samples) and air dried for 5 min. Slides were immersed in methanol at −20°C for 5 min. The wells were washed 10 times with 50 μl of 1% BSA in PBS. The primary antibody (15 μl of a 200- to 1,000-fold dilution) was applied to each well and incubated at room temperature for 2 h. Slides were further washed 10 times with 1% BSA in PBS. The fluorochrome-linked secondary antibody (500-fold dilution) was applied and incubated for 1 h at room temperature. The cells were again washed 10 times with PBS. Mounting medium was added and cover glasses were applied and sealed with nail polish. Cells were observed under a Zeiss Axiophot fluorescence microscope.

Table II. Yeast Strains Used in This Study

Name	Genotype	Source
GC1-8B	*MATa, leu2-3,112, ura3-1, trp1-1, ctt1-1, cta1-2*	(3)
DCT1-2C	*MATa, leu1, arg4, ctt1-1*	(3)
JW68-3A	*MATα, ura3-1, trp1-1, arg4, ctt1-1*	This study
m6-D1	*MATa, peb1-1, ura3-1, trp1-1, ctt1-1*	This study
m11-A1	*MATα, peb2-1, ura3-1, trp1-1, arg4, ctt1-1*	This study
m33-C2	*MATα, peb3-1, ura3-1, trp1-1, arg4, ctt1-1*	This study
2m1-A4	*MATα, peb4-1, leu2-3,112, ura3-1, trp1-1, arg4, ctt1-1*	This study
m34-A4	*MATa, peb5-1, leu1, trp1-1, arg4, ctt1-1*	This study
m24-C4	*MATα, peb1-2, trp1, ura3-1, ctt1-1*	This study
BQS20	*MATa, ura3-1, leu2::HIS3, pot1::URA3*	(16)

Induction of Peroxisomes

The following standard conditions were used to induce peroxisomes in all experiments unless indicated otherwise. Cells were precultured in YPD medium overnight, diluted into more YPD medium at 3×10^4 ml, and grown exponentially for 16 to 18 h to a final density of approximately 5×10^7 cells/ml. The cells were then inoculated into YPGO medium at a density of 5×10^6 and grown for 18 h at 30°C. Under these conditions, wild-type cells, which have a doubling time of ~6 h in YPGO, were still growing exponentially. Cells were collected by centrifugation.

Electron Microscopy

Morphology. Whole cells were prepared as described by McConnell et al. (22) and Stevens (36) with the following modifications. Cells were prefixed with 3% glutaraldehyde in 0.1 M sodium cacodylate (pH 7.3) for 1 to 2 h at 4°C. Fixation was continued with 4% potassium permanganate for 1 h. The cells were post-fixed in 2% osmium tetroxide, stained with 1.5% uranyl acetate for 2 to 12 h, dehydrated in ethanol, and embedded in Epon 812. Fixation, dehydration, and staining were performed on a rotator at room temperature. Sections were cut with a Reichert Ultracut E ultramicrotome and examined under a Hitachi 7000 electron microscope.

Cytochemistry for Catalase Activity. Whole cells were prefixed with 3% glutaraldehyde in 0.1 M sodium cacodylate (pH 7.3) for 1 h at 4°C (6) and incubated in 3, 3'-diaminobenzidine (DAB)[1] (2 mg/ml) as described by van Dijken et al. (43). After the DAB reaction, cells were fixed with 1.5% potassium permanganate. The cells were then stained with 1.5% uranyl acetate and processed as described above. In control experiments, 50 mM 3-amino-1,2,4-triazole was included in the DAB reaction to specifically inhibit catalase activity (8, 23).

Immunoelectron Microscopic Cytochemistry. Whole cells were processed as described by Slot and Geuze (33) and by van Tuinen and Riezman (44) with the following modifications. Cells were fixed as a suspension in 4% paraformaldehyde and 1% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.3) for 2 h at room temperature. After washing, cells were incubated in 1% sodium metaperiodate for 1 h. Free aldehydes were quenched with 50 mM NH_4Cl. Small pellets were dehydrated in ethanol at −20°C, embedded in Lowicryl K4M, and polymerized at −20°C under UV light for 24 h. Silver sections were mounted on formvar coated 200-mesh nickel grids and blocked with 1% BSA in PBS. The sections were incubated with the primary antibody for 1 h, washed with PBS, and then incubated for 1 h with 10 nM gold-protein A (1:50). After the immunoreaction, the sections were further stained with uranyl acetate followed by lead citrate and examined by EM.

Other Methods

Mutagenesis with 3% ethylmethane-sulfonate (EMS) was carried out (29) with 60% mortality. Techniques of yeast genetics such as cell mating, sporulation, and tetrad analysis were done according to the standard protocols of Sherman et al. (29). Proteins were separated by SDS-PAGE and immunoblotted according to standard protocol (14); antibodies on the blots were detected by chemiluminescence with a Western blot detection kit from Amersham Corp. (Arlington Heights, IL). Catalase was assayed as described previously (2).

Materials

Rabbit anti-yeast thiolase was kindly provided by Dr. Wolf Kunau (University of Bochum, Bochum, Germany). A rabbit antiserum against total peroxisomal proteins from *C. tropicalis* (No. 10-324) (34) cross-reacts with catalase and several proxisomal proteins of unknown function in *S. cerevisiae* (G. M. Small, personal communication). It does not cross-react with *S. cerevisiae* thiolase. FITC-conjugated goat anti-rabbit Ig was from Boehringer Mannheim Corp. (Mannheim, Germany). Texas red linked to sheep anti-rat Ig was from Amersham International plc (Buckinghamshire, England). Monoclonal rat anti-yeast-α-tubulin (YOL1/34) was from Accurate Chemical and Scientific Corp. (Westbury, NY). HRP-conjugated goat anti-rabbit Ig and enhanced chemiluminescence Western blotting detection reagents were from Amersham International plc. EMS (M 0880) was from Sigma Immunochemicals (St. Louis, MO). Zymolyase 100-T was from ICN Biomedicals, Inc. (Costa Mesa, CA).

Results

Toxicity of H_2O_2 Produced during Peroxisomal Oleate Metabolism in Catalase-lacking Yeast

We tested the hypothesis that H_2O_2 produced by the peroxisomal metabolism of fatty acids would be toxic to cells lacking the protection of catalase. Two related yeast strains, GC1-8B, which lacks both catalase A and catalase T, and DCT1-2C, which lacks only cytosolic catalase T (3), were precultured in oleate-containing medium to induce peroxisomes and then were spread on plates containing oleic acid plus glycerol as a carbon source (YPGO). The strain devoid of catalase activity was viable, but grew very slowly, forming tiny colonies after five days (Fig. 2 *B*). The strain containing peroxisomal catalase grew much faster and formed large colonies (Fig. 2 *A*). Both strains grew rapidly on plates containing glycerol alone (YPG) (Table III). This suggests that H_2O_2 formation is indeed toxic to cells lacking catalase.

The strain lacking catalase was mutagenized with 3% EMS, cultured for 24 h with oleate (YPOT), and spread on YPGO plates. Mutants unable to form peroxisomes were predicted to not make H_2O_2, and therefore to be healthy and grow well (Table I). As expected, a few large colonies were observed on a lawn of tiny colonies (Fig. 2 *C*). These data suggested that the proposed positive selection procedure was feasible.

Isolation of Peroxisome Biogenesis Mutants

Approximately 1×10^6 mutagenized cells were screened for the ability to grow rapidly (on ~100 YPGO plates as in Fig. 2 *C*). Nearly 1.7×10^3 large colonies were found on the background of numerous tiny colonies. These large colonies were candidates for peroxisome biogenesis (*peb*) mutants. We were aware, however, that large colonies could also have resulted from other mutations, such as structural defects in fatty acid oxidation enzymes. Therefore, a simplified immunofluorescence procedure (see Materials and Methods) was employed to screen the collection of large colonies for mutants which were deficient in peroxisome biogenesis. An antibody against thiolase, an enzyme that is normally located exclusively inside peroxisomes, was used for this purpose. It produced a punctate pattern of immunofluorescence in wild-type cells containing normal peroxisomes (Fig. 3 *A*).

Among the large colonies, 6.3% (107) had an abnormal thiolase fluorescence pattern. Some of these mutants showed thiolase fluorescence throughout the cytosol (Fig. 3 *C*). The fluorescence intensity of these cells appeared to be substantially greater than in wild-type cells for unknown reasons. Subsequent immunoblot analysis showed that the total amount of thiolase protein in these mutants was not significantly different from wild type (49). Other mutant phenotypes of thiolase immunofluorescence are described below.

Cloning and Rescreening

Cells from each of the 107 large colonies with abnormal thiolase immunofluorescence patterns were cloned and reanalyzed by immunofluorescence at least twice. As a positive

1. *Abbreviations used in this paper*: D, dextrose; EMS, ethylmethane-sulfonate; G, glycerol; N, yeast nitrogen base; O, oleic acid; P, bacto-peptone; pas, peroxisome assembly; peb, peroxisome biogenesis; T, Tween 40; Y, yeast extract.

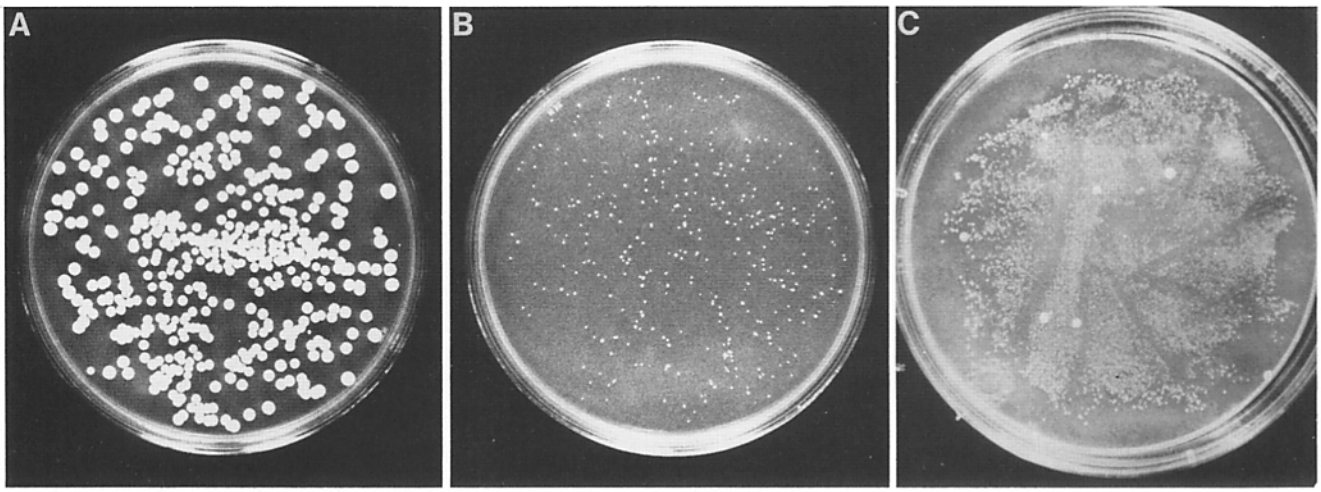


Figure 2. Growth of catalase-containing and catalase-deficient yeast in the presence of the fatty acid, oleate. (*A*) Catalase A-containing strain (DCT1-2C) (*B*) Catalase-deficient strain (GC1-8B). (*C*) Catalase-deficient strain after mutagenesis. The strains were precultured in YPD medium overnight, inoculated into YPOT medium at 1×10^6 ml, and grown for 24 h. Cells were then spread on YPGO plates (which contain both glycerol and oleate) and incubated for 5 d at 30°C. About 400 cells were put on plates *A* and *B* and about 10^4 cells on plate *C*. YPD preculture was omitted in *C*.

control, mAbs against tubulin were included (Fig. 3 *B* and *D*). 11 clones with reproducible and interesting mutant immunofluorescence phenotypes were chosen for further analysis.

The mutants were tested for their ability to grow on oleic acid as the sole carbon source (YNO plates), which was expected to require intact peroxisomes. The mutants with altered intracellular distributions of thiolase (as assessed by fluorescence) did not grow at all on YNO, whereas the parent strain (GC1-8B) grew slowly (data not shown).

Genetic Analysis

Each of the 11 mutant clones described above was mated with wild-type yeast. Diploids were tested for their ability to grow on oleate as the sole carbon source (YNO plates) and for peroxisome integrity (by immunofluorescence). All diploid cells grew on YNO plates and had wild-type thiolase fluorescence patterns (data not shown), indicating that the mutations in these 11 clones are recessive.

The diploids were sporulated and 10–12 tetrads from each cross were dissected. All showed a 2:2 segregation pattern for growth on YNO. Three or four tetrads per cross were further analyzed by immunofluorescence and all showed 2:2 segregation for punctate, wild-type thiolase immunofluorescence. A typical result is illustrated in Fig. 4: two of the four meiotic products have cytosolic thiolase fluorescence and also fail to grow on YNO; the other two progeny have punctate immunofluorescence and do grow on YNO. These results show that each of the 11 mutant phenotypes is caused by a single gene mutation.

*Table III. Growth of Yeast Strains with or without Fatty Acids in the Medium**

Yeast strain	DCT1-2C	GC1-8B
Cytosolic catalase T	No	No
Peroxisomal catalase A	Yes	No
Growth on glycerol	Large colonies	Large colonies
Growth on glycerol plus oleic acid	Large colonies	Tiny colonies

* The strains were precultured in YPD medium, grown for 24 h in YPOT medium, and spread on YPG or YPGO plates. The plates were incubated for 5 d.

Peb mutants with appropriate mating types and genetic markers (selected from the tetrads) were used for complementation studies. Complementation was assessed by growth on YNO medium and by immunofluorescence. By both criteria, the 11 mutants fell into five complementation groups (Table IV).

From each group, one mutant was chosen for further analysis. Mutant strains that contained catalase A were selected, in order that the packaging of this enzyme could be tested. The gene encoding catalase A came from the wild-type strain with which the mutants had been backcrossed during the genetic analysis described above.

Immunofluorescence Patterns in the Five Peb Mutants

In the studies reported here and in the rest of this paper, cells were routinely grown for 18 h in a medium containing both glycerol and oleic acid as carbon sources.

Peroxisomes were readily visualized by immunofluorescence with anti-thiolase in wild-type yeast grown on YPGO. They appeared, in a typical focal plane, as 4–12 punctate fluorescence structures, usually located near the cell plasma membrane (Fig. 5). No appreciable cytosolic fluorescence was seen in wild-type cells.

In three of the five new yeast mutant complementation groups (*pebl*, *peb2*, and *peb4*), the cell cytosol was filled with strong fluorescence and no punctate structures were seen (Fig. 5). This indicates that thiolase was expressed in the mutants, but was not packaged into peroxisomes. The presence or absence of peroxisomes and the packaging of other peroxisomal proteins was examined by EM and immunolabeling, as described below.

In one new complementation group, *peb5*, most cells con-

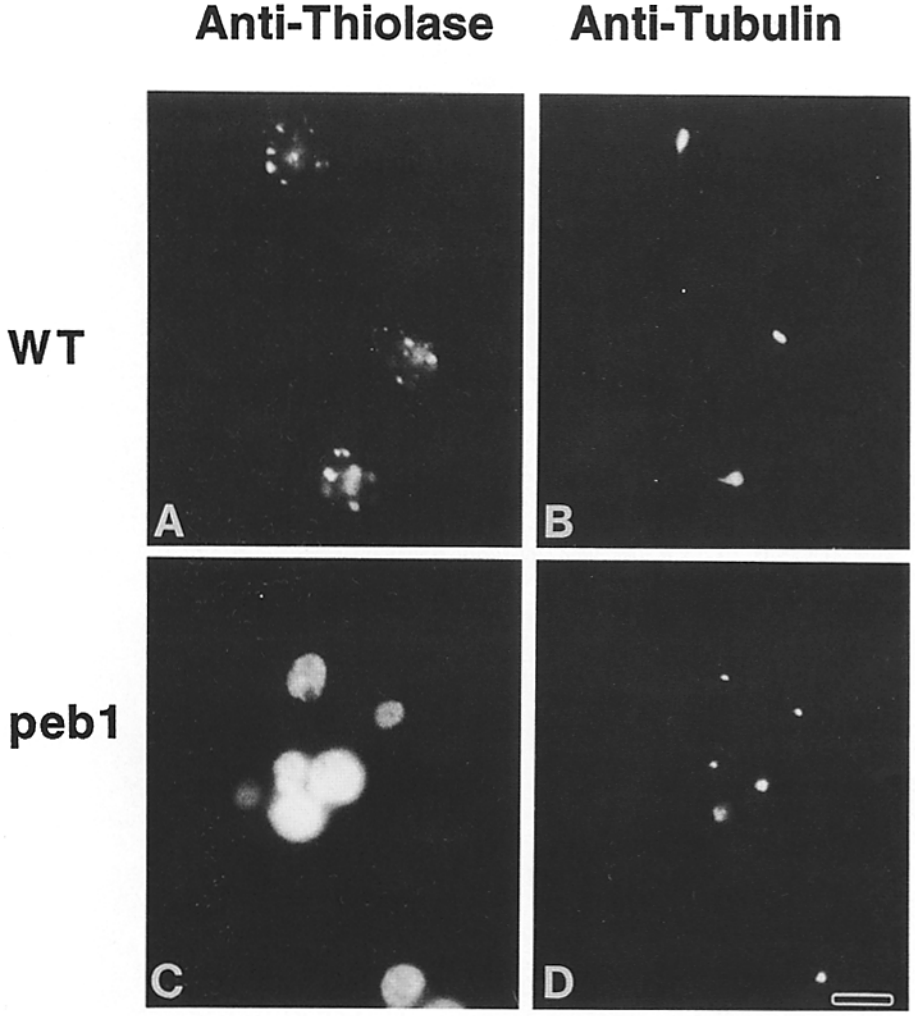


Figure 3. Simplified double immunofluorescence of yeast cells in order to analyze thiolase with a tubulin control. Yeast colonies (50–100 at a time) were converted to spheroplasts, lightly fixed, and permeabilized as described in Materials and Methods. A mixture of rabbit antiserum against yeast thiolase (*A* and *C*) and monoclonal rat antibody against yeast tubulin (*B* and *D*) was applied. The secondary antibodies were goat anti-rabbit IgG conjugated with FITC and sheep anti-rat IgG conjugated with Texas red. Wild-type observed sequentially for thiolase (*A*) and tubulin (*B*). Mutant cells, m6, observed sequentially for thiolase (*C*) and tubulin (*D*). Bar, 5 μm.

tained one or two strongly fluorescent structures that were distinctly larger than normal peroxisomes (Fig. 5). These cells also contained some fluorescent particles the size of normal peroxisomes. The huge fluorescent particles could be giant peroxisomes or clusters of peroxisomes, or perhaps could be due to the mislocalization of thiolase into vacuoles.

In the *peb3* complementation group, cells contained punctate fluorescent structures, but the fluorescence intensity was much fainter than in wild-type cells (Fig. 5). The number of particles also appeared to be less than that of wild-type cells. This might result from the reduced expression of thiolase and perhaps other peroxisomal proteins. Many other mutants detected in the initial screen showed this fluorescence phenotype; since it appeared likely that they represent regulation mutants, most of them were set aside for the time being.

Mutants Lacking Recognizable Peroxisomes

Peb2—Electron Microscopy. Mutant m11-A1 had a nearly normal appearance when grown on YPGO and examined by EM (Fig. 6, *peb2*). The nucleus, mitochondria, and vacuole were present and demonstrated their usual ultrastructural appearances. Most of the ER was located adjacent to the plasma membrane. White (electron transmitting) droplets of lipid were frequently found in wild-type and *peb2* cells. They result from the yeast having taken up fatty acid from the medium and stored it in droplets. No peroxisomes were found in *peb2* cells, despite careful examination of hundreds of sections. This contrasts with wild-type cells grown in YPGO, in which peroxisomes were observed as individual, round structures scattered through the cytoplasm. In wild-type yeast, the peroxisomes were usually somewhat smaller than mitochondria (Fig. 6).

Peb2—Electron Microscopic Cytochemistry. In a further effort to find peroxisomes in *peb2* mutant cells, we employed a cytochemical reaction in which an electron-dense deposit of oxidized DAB is deposited on structures containing catalase (43). This causes wild-type peroxisomes to stand out as darkly stained circles that are easy to identify (Fig. 7, *WT*). No peroxisomes were found in hundreds of sections of *peb2* cells (Fig. 7).

Catalase is present in *peb2* at approximately normal levels. It is probably mislocalized to the cytosol, where the concentration would be much lower than within peroxisomes, which would explain the lack of demonstrable DAB staining. Cytosolic catalase in Zellweger hepatocytes, likewise, does not produce noticeable cytosolic DAB reaction product (9).

Peb4. Mutant 2m1, *peb4*, also lacked peroxisomes, based on EM (Fig. 6) and electron microscopic cytochemistry

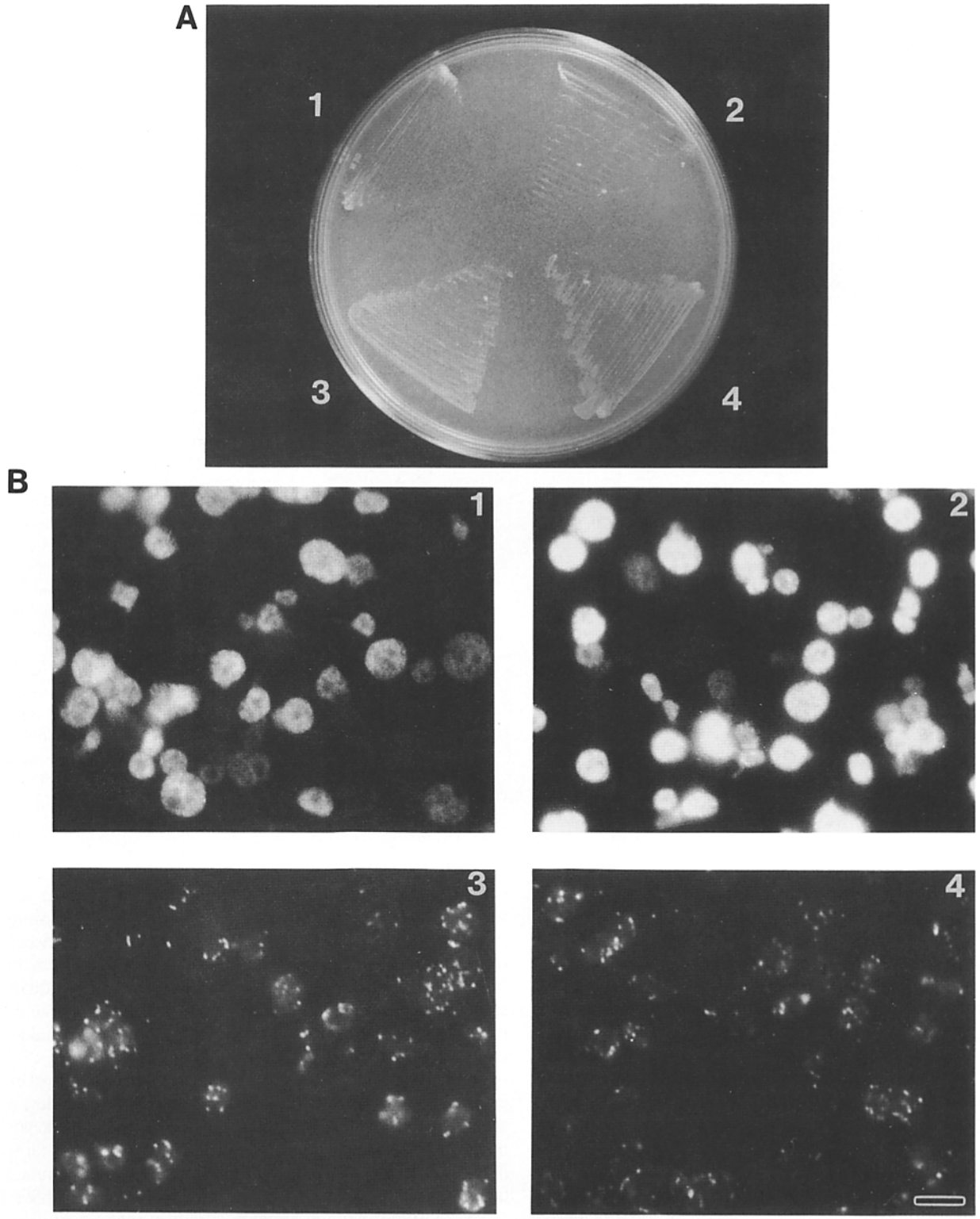


Figure 4. Genetic analysis of peroxisome biogenesis mutants. Mutant 2ml was mated with wild-type JW68-3A, diploid cells were sporulated, and tetrads were dissected. (*A*) Growth of the four spores from one tetrad on YNO plates. (*B*) Immunofluorescence of the same four meiotic progeny with antiserum against thiolase. Bar, 5 μm.

Table IV. Mutant Phenotypes and Complementation

Mutant	Thiolase immunolocalization pattern	Growth on YNO	*Peb* complementation group*
m6	Cytosolic	–	1
m13	Cytosolic	–	1
m24	Cytosolic	–	1
m28	Cytosolic	–	1
m29	Cytosolic	–	1
m11	Cytosolic	–	2
m33	Weak particles	–	3
m9	Weak particles	–	3
2m1	Cytosolic	–	4
m34	Large particles	–	5

* All mutations were recessive and segregated 2:2.

(Fig. 7). The cytoplasm of this mutant consistently appeared abnormal, with large vacuoles and lipids droplets. Nevertheless, it grew as rapidly as *peb2*. Other organelles such as mitochondria and ER were recognizable, and catalase activity was normal. This mutant must be backcrossed further with wild-type yeast in the future in order to test whether there is a connection between the absence of peroxisomes and the other morphological changes.

Peroxisome Membrane Ghosts? *Peb2* and *peb4* have a phenotype similar to Zellweger cells, in that normal-looking peroxisomes are missing. It has not yet been possible to test whether *peb2* and *peb4* cells have largely empty membrane ghosts of peroxisomes, such as are seen in Zellweger cells, because of the lack of a suitable antibody. However, one image of *peb4* showed curious membrane structures that could be candidates for peroxisome ghosts (Fig. 8). No such structures have been detected thus far in *peb2*.

Complementation with Kunau's Pas Mutants. Mutant 2m1 was mated with each of the peroxisome assembly (*pas*) mutants 1–6 (6, 18). In these six *pas* mutants, peroxisomal proteins, including thiolase, are mislocalized to the cytosol. In each mating, the resulting diploids showed wild-type punctate thiolase fluorescence. Therefore, *peb4* belongs to a different complementation group than *pas1–6*. On the other hand, mutant m11 (*peb2*) failed to complement *pas1*, suggesting that m11 is an allele of *pas1*.

Mutants in Which Peroxisomes Are Present in Clusters and Packaging of Peroxisomal Enzymes Is Selectively Defective

Peb1. Mutant m6-1, *peb1*, showed strong cytosolic fluorescence with anti-thiolase, just like *peb2* and *peb4*. However, *peb1* contained normal-looking peroxisomes by EM (Fig. 6). Instead of being distributed throughout the cytosol, the peroxisomes occurred in clusters. An example of a cluster of four peroxisomes in *peb1* is shown in Fig. 6. Although clusters of peroxisomes were occasionally found in wild-type yeast grown in YPGO, clustering was consistently observed in *peb1*.

Catalase is packaged normally into peroxisomes in *peb1*, as demonstrated by cytochemical staining. A cluster of seven DAB-positive peroxisomes is illustrated in Fig. 7 (*peb1 panel*).

The inability of this mutant to package thiolase into peroxisomes, shown by the immunofluorescence data, was tested further by immunogold labeling. As shown in Fig. 9, wild-type yeast peroxisomes, reacted with anti-thiolase, contained abundant gold particles (*arrows*). In contrast, peroxisomes in *peb1* were unlabeled. Thus this mutant has a selective defect in peroxisomal protein packaging: the clustered peroxisomes can import catalase but not thiolase.

Thiolase Targeting Information Is Intact in Peb1. The inability of the *peb1* mutant to package thiolase could be the result of a mutation in the thiolase gene that abolishes the targeting information that directs thiolase to peroxisomes. Alternatively, it could be due to a mutation in a gene that encodes a protein that is required to import thiolase, but not catalase, into peroxisomes. These possibilities were tested by mating *peb1–2* with a yeast strain in which the thiolase gene had been knocked out (16). In the thiolase knockout strain, no thiolase protein was detected by immunoblotting (Fig. 10 *C*, lane *2*) or by immunofluorescence (Fig. 10 *A*, panel *2*). Like *peb1*, this strain did not grow on YNO (Fig. 10 *B*, sectors *1* and *2*). The diploid resulting from the cross between this strain and *peb1–2* demonstrated a wild-type pattern of thiolase immunofluorescence (Fig. 10 *A*, panel *3*). Moreover, the diploid grew on YNO (Fig. 10 *B*, sector *3*), indicating regain of peroxisomal function. These data demonstrate that the thiolase in *peb1* contains the necessary targeting information to be packaged into peroxisomes. Therefore, *peb1* must contain a mutation in machinery that is specifically necessary for thiolase import.

Immunoelectron microscopy was also carried out with an antibody that recognizes several peroxisomal proteins other than thiolase (see Materials and Methods). It gave a strong gold labeling of wild-type peroxisomes, and a reduced labeling of peroxisomes in *peb1* (Fig. 11). This suggests that thiolase is probably not the only peroxisomal enzyme whose packaging is impaired in *peb1*.

peb5. In mutant m34, *peb5*, thiolase appeared to be in huge structures according to the immunofluorescence data (Fig. 5). By EM analysis, peroxisomes of normal size were present in *peb5*. As in *peb1*, these peroxisomes were consistently observed in clusters (Fig. 6). Immunoelectron microscopy of these peroxisomes demonstrated that they contain thiolase (Fig. 9). Therefore, the large fluorescent particles seen in Fig. 5 are in fact clusters of normal-sized peroxisomes.

These clustered peroxisomes do not contain catalase, according to EM cytochemistry. A cluster of seven DAB-negative peroxisomes is shown in Fig. 7 (*peb5 panel*). It is noteworthy that electron dense DAB was observed in vacuoles in *peb5*. However, this DAB deposition was also seen in cytochemical controls in which catalase activity was inhibited with aminotriazole (Fig. 7, *peb5 inset*). Therefore, the vacuolar DAB is due to some cause other than catalase enzyme activity. Similar staining of mammalian lysosomes by DAB in a catalase-independent, nonenzymatic fashion has sometimes been observed (8, 23).

The activity of catalase in *peb5* was approximately the same as in wild-type cells. The absence of DAB reactivity in the peroxisomes or in any other organelle in *peb5* suggests that the catalase is cytosolic. Mislocalization of catalase to the cytosol may also explain the reduced staining of mitochondrial cristae by DAB that was observed in *peb5*. The

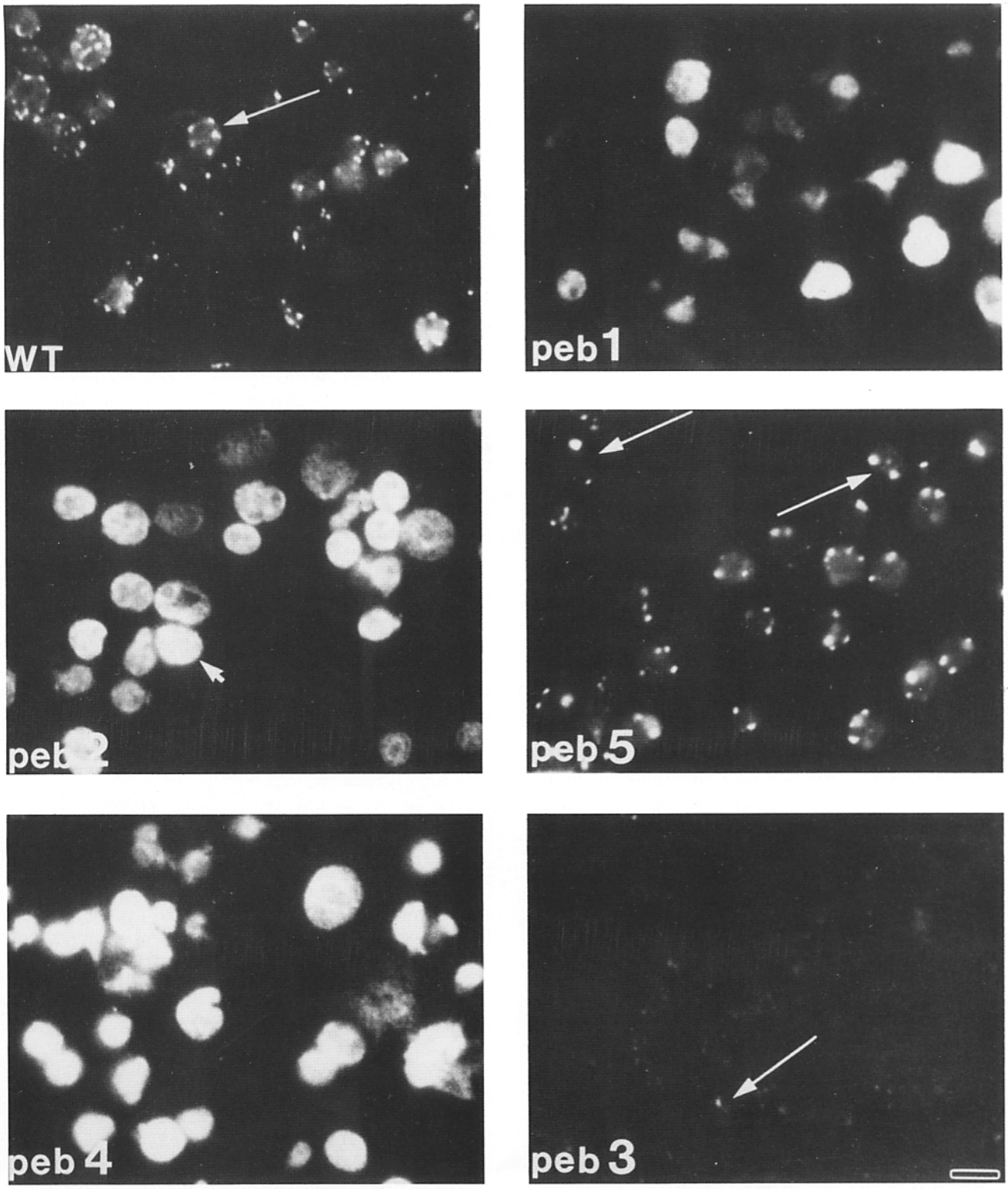
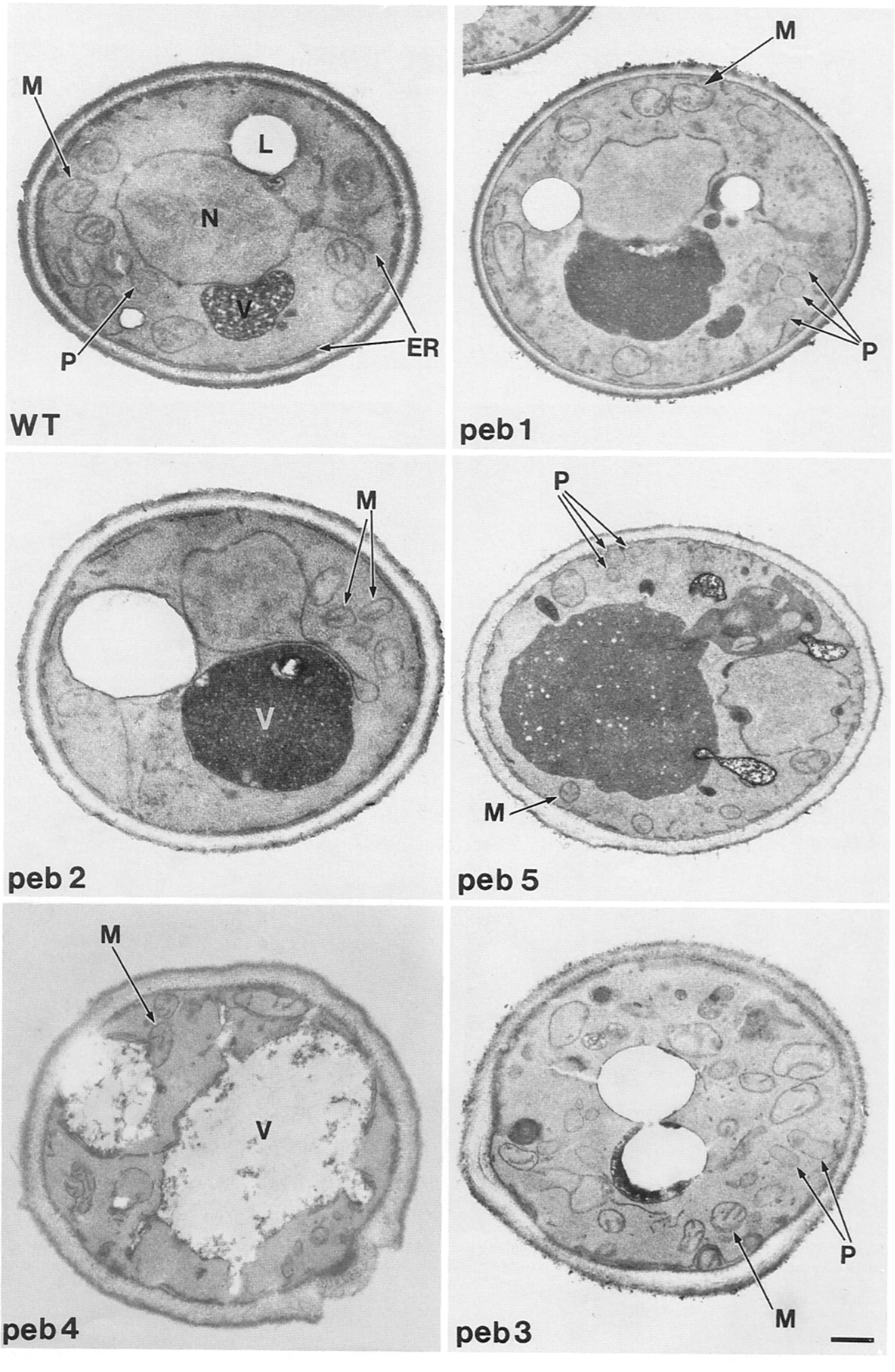


Figure 5. Immunofluorescence analysis of one mutant from each of the five *peb* complementation groups and of wild-type cells (*WT*) with anti-thiolase. Arrows indicate cells with typical appearance, discussed in the text. *peb 1* and *peb 2* cells that appear to have little or no fluorescence are mostly above or below the focal plane. The yeast were grown in YPGO for 18 h. Bar, 5 μm.

Figure 6. Electron microscopy of each of the five *peb* mutants and wild-type cells (*WT*). *ER*, endoplasmic reticulum. *L*, lipid droplet. *M*, Mitochondrion. *N*, Nucleus. *P*, Peroxisome. *V*, Vacuole. Note the presence of clusters of peroxisomes in mutants carrying the *peb1-1* and *peb5-1* mutations. Growth was in YPGO for 18 h. Bar, 0.4 μm.


M
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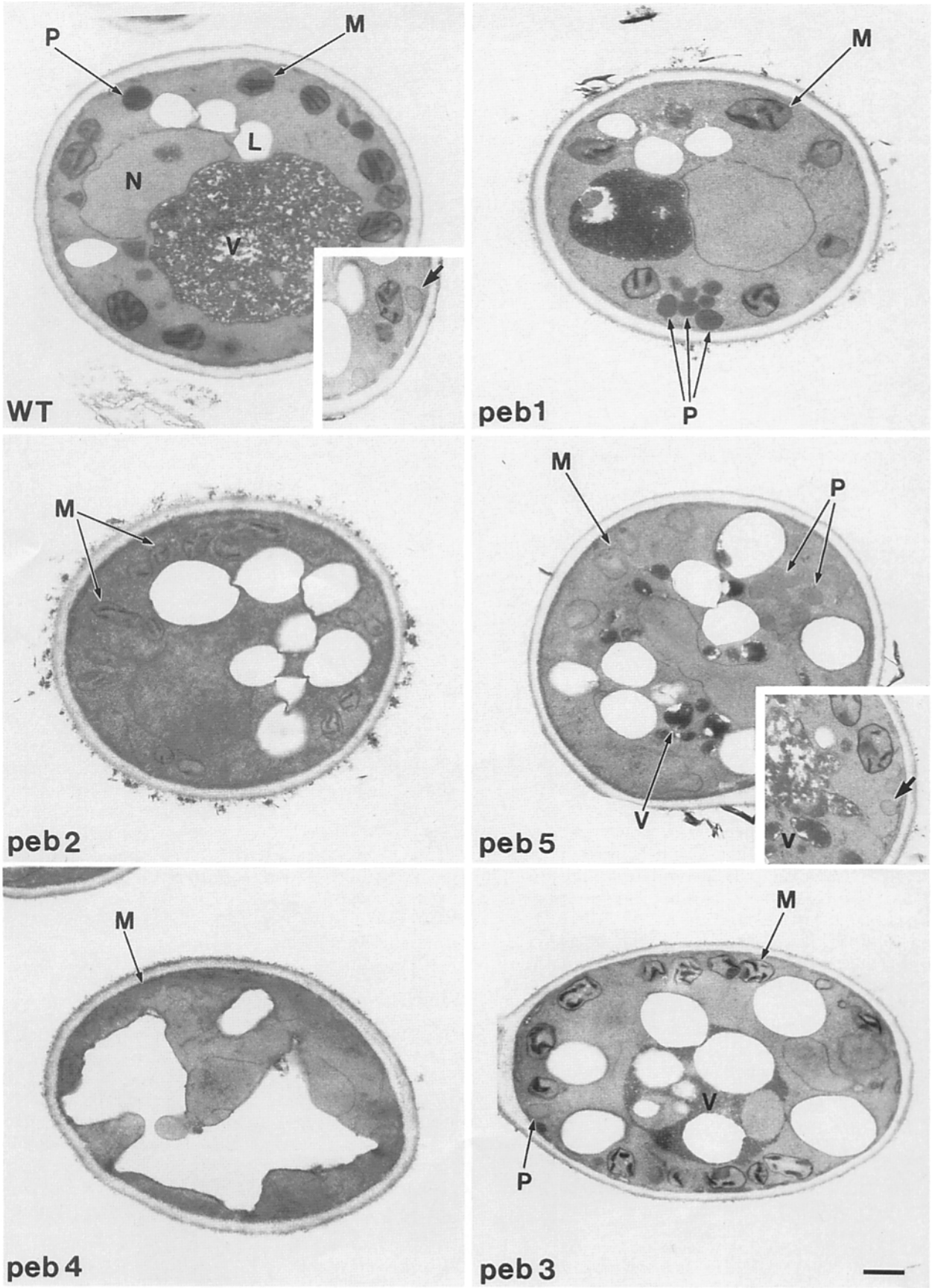

Figure 7. Cytochemical staining of catalase activity in the five *peb* mutants and wild-type cells (*WT*). These strains contain a wild-type catalase A gene which was introduced during the backcrossing of the mutants with wild-type yeast. Electron-dense, oxidized diaminobenzi-

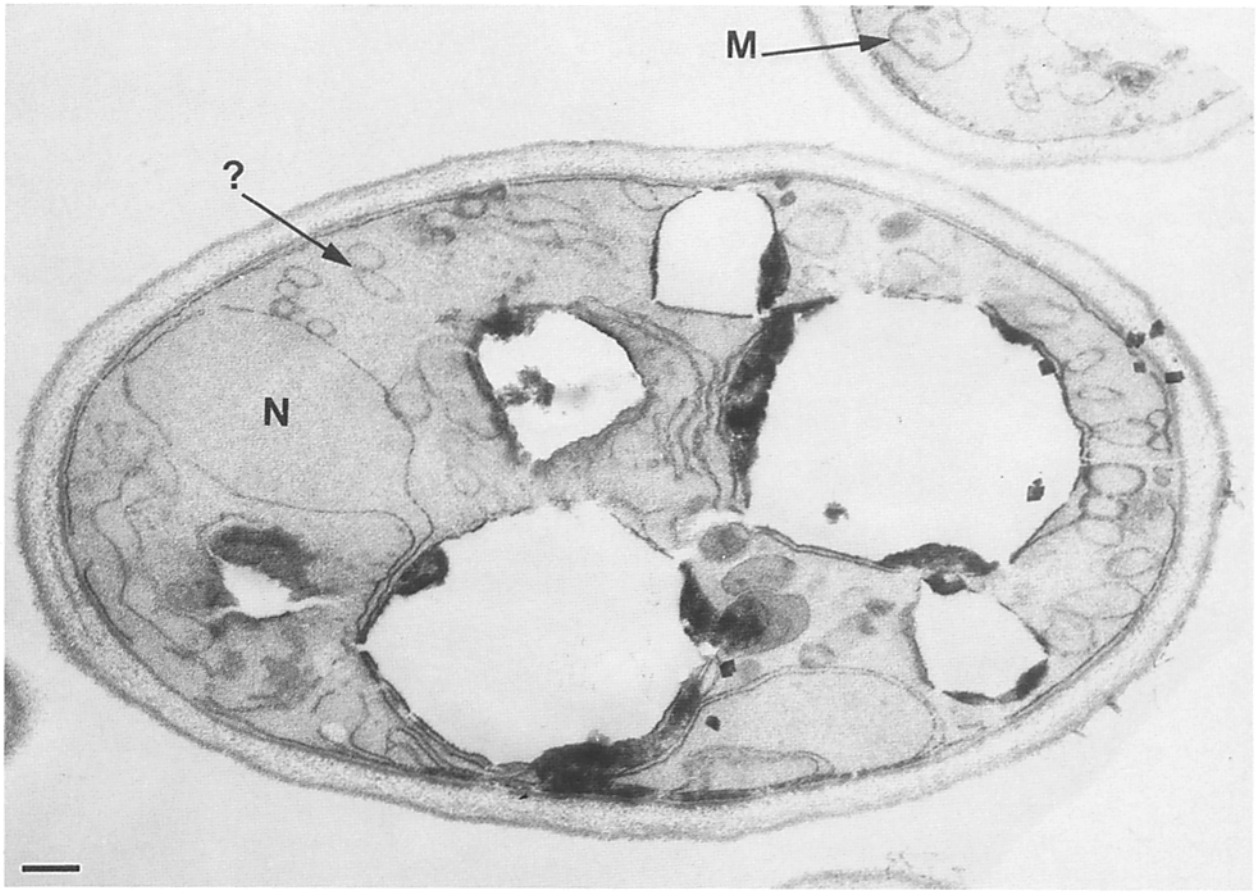

Figure 8. "Ghost" candidates (?) in *peb4*. Bar, 0.25 μm.

cytosolic catalase probably reduces the amount of externally added H_2O_2 that reaches the mitochondria during the cytochemical reaction. This interpretation is supported by the fact that when catalase was inhibited with aminotriazole, mitochondrial staining appeared (Fig. 7, *inset, peb5 panel*).

A wild-type catalase gene had been introduced into this *peb5* mutant by backcrossing. Therefore, the inability to import catalase into peroxisomes must be due to a defect in catalase-specific import machinery.

Immunogold labeling of peroxisomes with the antibody against several peroxisomal proteins was strikingly reduced in *peb5* relative to wild type (Fig. 11), suggesting that the packaging of additional proteins may also be impaired.

Both mutants m6 and m34 complemented all of the *pas* mutants 1–6. Therefore, *pebl* and *peb5* are different complementation groups, as might be expected from the different phenotypes.

A Mutant in Which the Peroxisomes Contain Less of Several Proteins

Peb3. Mutant m33, *peb3*, showed weak fluorescent particles with anti-thiolase (Fig. 5). This mutant contained peroxisomes (Fig. 6), which appeared to be somewhat less abundant than in wild-type cells. They did not show demonstrable DAB staining for catalase (Fig. 7). By immuno electron microscopy they demonstrated less immunoreactivity for thiolase than wild-type cells (Fig. 9) and no immunoreactivity with the antibody against several other peroxisomal proteins (Fig. 11). The abundance of these enzymes was also less than in wild-type cells according to immunoblot analyses (49). Thus, *peb3* may involve a subnormal induction of peroxisomal proteins by oleate.

Discussion

The positive selection procedure used here, which exploited the toxicity of hydrogen peroxide, led to the identification of 5 *peb* complementation groups. Four of these are distinct from all of the previously described peroxisome assembly mutants, *pasl–6*, of (6). This illustrates the rule that different selection strategies often yield different mutants. In the present case, the difference in strategy was subtle: Kunau screened for mutants that were unable to utilize fatty acid as sole carbon source, whereas we selected against mutants that uti-

dine is deposited on peroxisomes containing catalase, and also on mitochondrial cristae, due to an unrelated enzyme activity. Insets in *WT* and *peb5* show controls in which aminotriazole was included in the cytochemical reaction to specifically inhibit catalase activity; note the unstained peroxisomes (*short arrows*) and stained cristae. The intensity of cristae staining is lower in mutants in which there is a lot of cytosolic catalase, presumably because it prevents much of the externally added H_2O_2 from reaching mitochondria. Abbreviations are the same as in Fig. 6. Growth was in YPGO for 18 h. Bar, 0.4 μm; same magnification in insets.

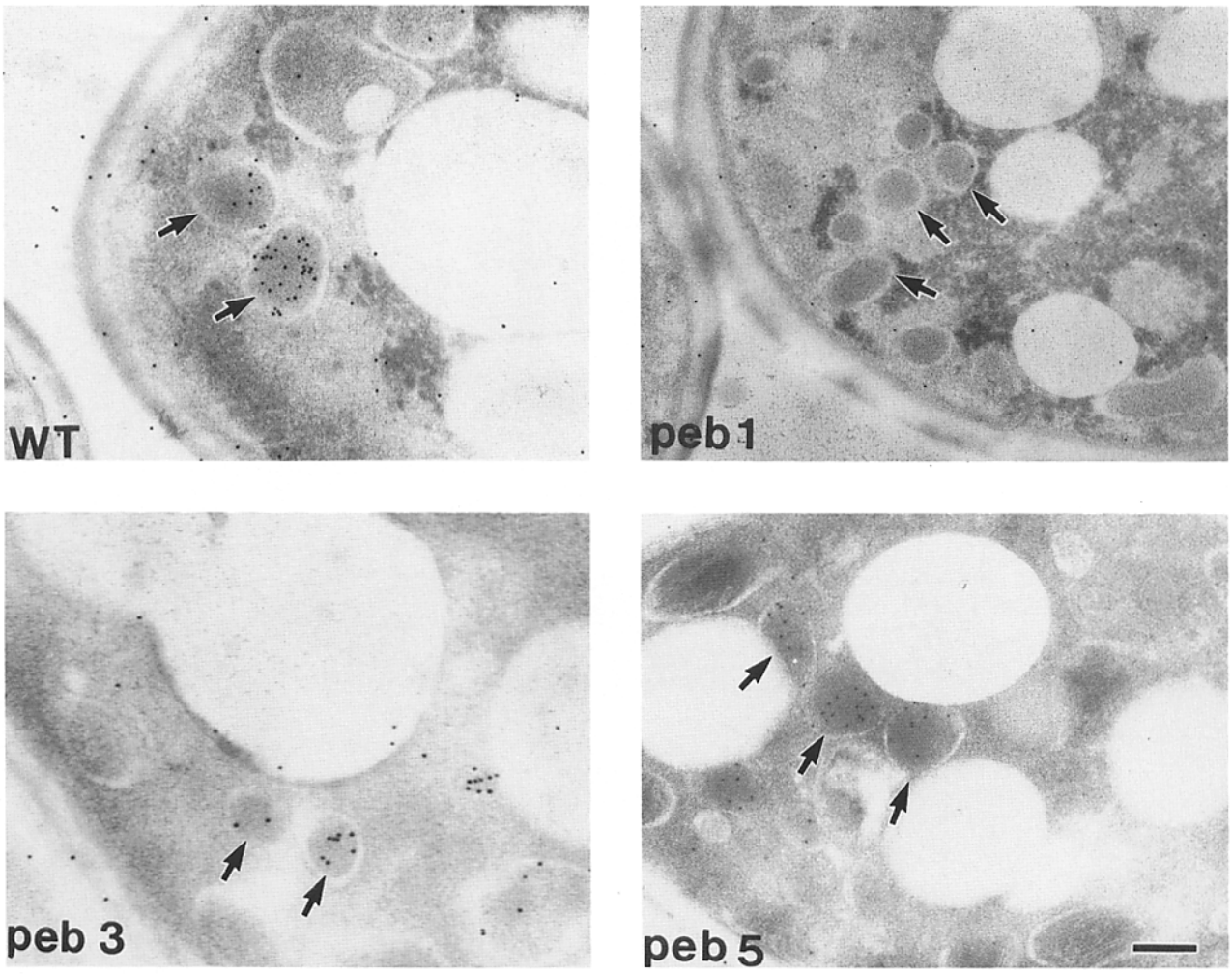

Figure 9. Immunoelectron microscopy to detect thiolase in wild-type cells and in the three *peb* mutants in which peroxisomes were found. Sections were incubated with rabbit antiserum against thiolase, followed by gold-conjugated protein A. Arrows indicate peroxisomes. Bar, 0.2 μm.

lized fatty acid in the presence of glycerol. Van Der Leij et al. (42) recently reported the isolation of some peroxisome assembly mutants by a variant of this procedure in which catalase was inhibited. There has not yet been an opportunity to cross-complement the mutants of the two labs. None of their mutants show the peroxisome clustering that we have observed, and our mutants did not have the reticular structure found in theirs.

Three of our complementation groups consisted of one mutant each, clearly suggesting that the search for peroxisome biogenesis mutants in *S. cerevisiae* is still not completed. The simplified immunofluorescence procedure introduced in this paper makes it possible to rapidly look for additional mutants in which individual peroxisomal proteins are incorrectly packaged. With the fluorescence screen one can avoid mutants in which the expression of peroxisomal proteins appears to be down-regulated which, while interesting, are not the focus of our current interest.

One of the most interesting aspects of our results is the unexpected observation that peroxisomes occur in distinct clusters in two of the mutants. There are many possible explanations. One speculation is that these peroxisomes might actually be interconnected. New peroxisomes form by division from preexisting peroxisomes (19), and if a protein that is required to finish the process of pinching off daughter peroxisomes were missing, the result might be an interconnected cluster, looking like a bunch of grapes. We plan to investigate this possibility in the future by serial sectioning.

Another unexpected result was that two of the mutants were selectively defective for the import of peroxisomal proteins. *Pebl* can import catalase but not thiolase. *Peb5* can import thiolase but not catalase. These partial packaging defects may be related to the existence of multiple types of targeting information that direct proteins to peroxisomes. Many proteins employ a carboxy-terminal SKL tripeptide, (10) whereas rat liver thiolase uses a cleavable amino-terminal oligopeptide (24, 38). In contrast, *C. tropicalis* acyl-CoA oxidase uses two redundant internal sequences

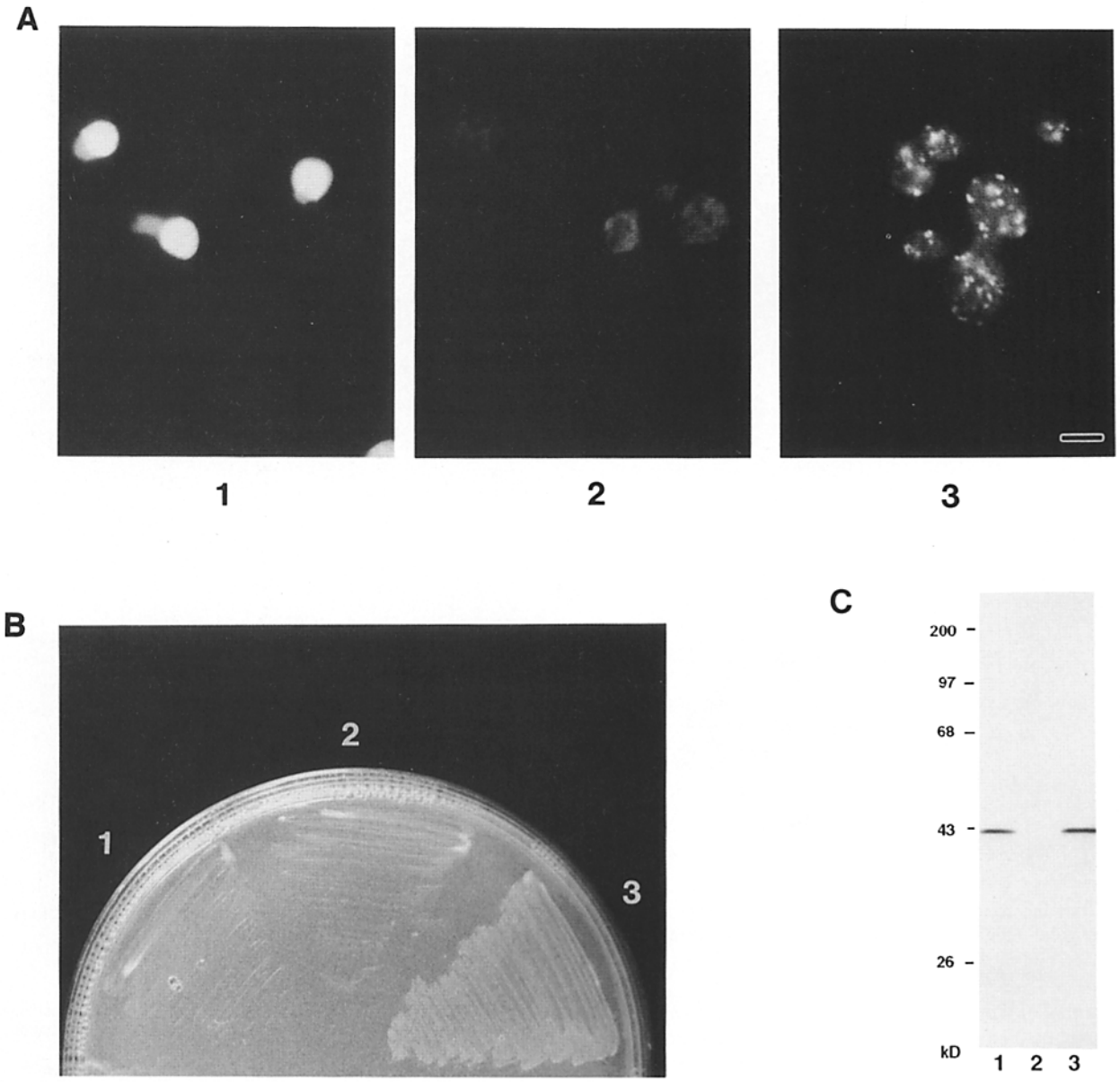

Figure 10. Thiolase in the *pebl* mutant has functional targeting information. *A* (1–3) Immunofluorescence analysis with anti-thiolase of: (1) *pebl-2* (m24-C2), (2) strain BQS20 in which the thiolase gene had been knocked out, and (3) the diploid formed by mating *pebl-2* with BQS20. *B* (1–3) growth on YNO plates. *C* (1–3) immunoblot analysis with antithiolase.

(35). *S. cerevisiae* catalase A uses an internal sequence and a redundant carboxy-terminal SSNSKF (17). It is not yet known what kind of targeting information is used by *S. cerevisiae* thiolase, but if we assume that in *S. cerevisiae*, thiolase and catalase are directed to peroxisomes by different classes of targeting information, then we may speculate that specific receptors for these targeting sequences might be individually mutated. In *pebl* a receptor used by thiolase might be defective. In *peb5* a receptor used by catalase might be defective. We have established an in vitro import assay for *S. cerevisiae* peroxisomes (39) with which these hypotheses may be tested.

It is noteworthy that *pebl* and *peb5* have different partial packaging defects, but both show peroxisome clustering. Since these are both due to single gene defects, we speculate that there must be two independent proteins that are required for pinching off new peroxisomes: one would have a targeting signal in common with catalase and the other a targeting signal in common with thiolase.

Two of our yeast mutants resemble human Zellweger syndrome in that recognizable peroxisomes are not detectable. In all of the nine Zellweger complementation groups that have been identified to date, fibroblasts contain peroxisomal ghosts (26, 28, 37, 46); thus, these are import mutants. If our

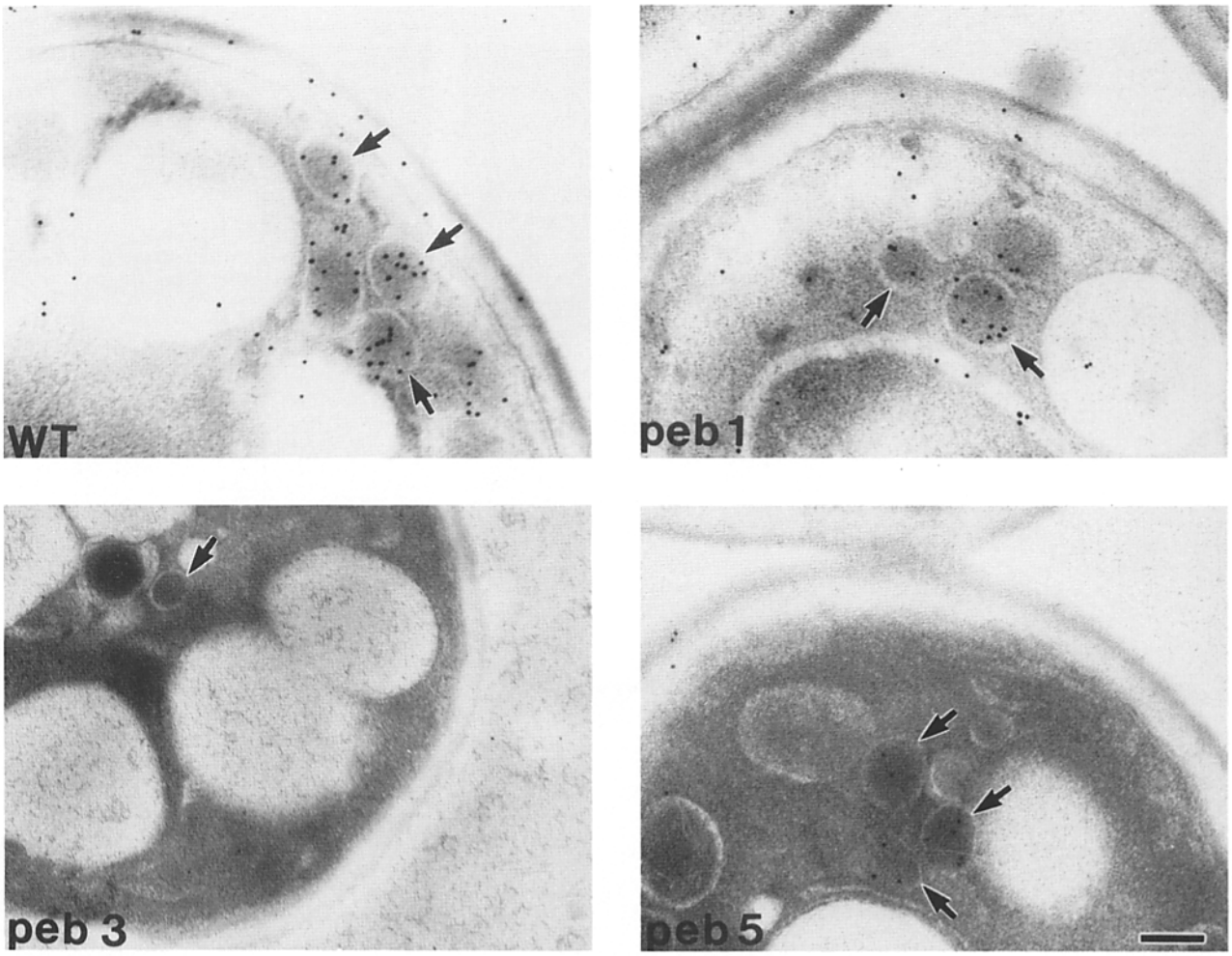

Figure 11. Immunoelectron microscopy with a rabbit antiserum that reacts with several peroxisomal proteins (see Materials and Methods) followed by gold-conjugated protein A. The cell samples are the same as in Fig. 9. Arrows indicate peroxisomes. Bar, 0.2 μm.

yeast mutants contain ghosts, analysis of the defective gene products should shed light on the process by which proteins are imported into peroxisomes. If the yeast mutants lack ghosts, they may shed light on the manner of assembly of the peroxisomal membrane itself.

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References

1. Aitchison, J. D., W. W. Murray, and R. A. Rachubinski. 1991. The carboxyl-terminal tripeptide Ala-Lys-Ile is essential for targeting *Candida tropicalis* trifunctional enzyme to yeast peroxisomes. *J. Biol. Chem.* 266:23197–23203.
2. Baudhuin, P., H. Beaufay, Y. Rahman-Li, O. Z. Sellinger, R. Wattiaux, P. Jacques, and C. de Duve. 1964. Tissue fractionation studies. 17. Intracellular distribution of monoamine oxidase, aspartate aminotransferase, alanine aminotransferase, D-amino acid oxidase and catalase in rat-liver tissue. *Biochem. J.* 92:179–184.
3. Cohen, G., F. Fessl, A. Traczyk, J. Rytka, and H. Ruis. 1985. Isolation of the catalase A gene of *Saccharomyces cerevisiae* by complementation of the *cta1* mutation. *Mol. Gen. Genet.* 200:74–79.
4. Cregg, J. M., I. J. Van Klei, G. J. Sulter, M. Veenhuis, and W. Harder. 1990. Peroxisome-deficient mutants of *Hanseluna polymorpha*. *Yeast.* 6:87–97.
5. Didion, T., and R. Roggenkamp. 1990. Deficiency of peroxisome assembly in a mutant of the methylotrophic yeast *Hansenula polymorpha*. *Curr. Genet.* 17:113–117.
6. Erdmann, R., M. Veenhuis, D. Mertens, and W.-H. Kunau. 1989. Isolation of peroxisome-deficient mutants of *Saccharomyces cerevisiae*. *Proc. Natl. Acad. Sci. USA.* 86:5419–5423.
7. Erdmann, R., F. F. Wiebel, A. Flessau, J. Rytka, A. Beyer, K.-U. Frohlich, and W.-H. Kunau. 1991. *PAS1*, a yeast gene required for peroxisome biogenesis, encodes a member of a novel family of putative ATPases. *Cell.* 64:499–510.
8. Fahimi, H. D. 1969. Cytochemical localization of peroxidatic activity of catalase in rat hepatic microbodies (peroxisomes). *J. Cell Biol.* 43: 275–288.
9. Goldfischer, S., C. L. Moore, A. B. Johnson, A. J. Spiro, M. P. Valsamis, H. K. Wisniewski, R. H. Ritch, W. T. Norton, I. Rapin, and L. M. Gartner. 1973. Peroxisomal and mitochondrial defects in the cerebro-hepato-renal syndrome. *Science (Wash. DC).* 182:62–64.
10. Gould, S. J., G.-A. Keller, N. Hosken, J. Wilkinson, and S. Subramani. 1989. A conserved tripeptide sorts proteins to peroxisomes. *J. Cell Biol.* 108:1657–1664.
11. Gould S. J., G.-A. Keller, M. Schneider, S. H. Howell, L. J. Garrard, J. M. Goodman, B. Distel, H. Tabak, and S. Subramani. 1990. Peroxisomal protein import is conserved between yeast, plants, insects and mammals. *EMBO (Eur. Mol. Biol. Organ.) J.* 9:85–90.
12. Gould, S. J., S. Krisans, G.-A. Keller, and S. Subramani. 1990. Antibodies directed against the peroxisomal targeting signal of firefly luciferase

recognize multiple mammalian peroxisomal proteins. *J. Cell Biol.* 110:27–34.
13. Gould, S. J., D. McCollum, A. P. Spong, J. A. Heyman, and S. Subramani. 1992. Development of the yeast *Pichia pastoris* as a model organism for the genetic and molecular analysis of peroxisome assembly. *Yeast.* 8:613–628.
14. Harlow, E., and D. Lane. 1988. Antibodies. A Laboratory Manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York. 421 pp.
15. Hohfeld, J., M. Veenhuis, and W.-H. Kunau. 1991. PAS3, a *Saccharomyces cerevisiae* gene encoding a peroxisomal integral membrane protein essential for peroxisome biogenesis. *J. Cell Biol.* 114: 1167–1178.
16. Igual, J. C., E. Matallana, C. Gonzalez-Bosch, L. Franco, and J. E. Perez-Ortin. 1991. A new glucose-repressible gene identified from the analysis of chromatin structure in deletion mutants of yeast SUC2 locus. *Yeast.* 7:379–389.
17. Kragler, F., A. Langeder, J. Raupachova, M. Binder, and A. Hartig. 1983. Two independent peroxisomal targeting signals in catalase A of *Saccharomyces cerevisiae. J. Cell Biol.* 120:665–673.
18. Kunau, W.-H., and A. Hartig. 1992. Peroxisome biogenesis in *Saccharomyces cerevisiae. Antonie Leeuwenhoek.* 62:63–78.
19. Lazarow, P. B., and Y. Fujiki. 1985. Biogenesis of peroxisomes. *Annu. Rev. Cell Biol.* 1:489–530.
20. Liu, H., X. Tan, M. Veenhuis, D. McCollum, and J. M. Cregg. 1992. An efficient screen for peroxisome-deficient mutant of *Pichia pastoris. J. Bacteriol.* 174:4943–4951.
21. McCammon, M. T., M. Veenhuis, S. B. Trapp, and J. M. Goodman. 1990. Association of glyoxylate and beta-oxidation enzymes with peroxisomes of *Saccharomyces cerevisiae. J. Bacteriol.* 172:5816–5827.
22. McConnell, S. J., L. C. Stewart, A. Talin, and M. P. Yaffe. 1990. Temperature-sensitive yeast mutants defective in mitochondrial inheritance. *J. Cell Biol.* 111:967–976.
23. Novikoff, A. B., and S. Goldfischer. 1969. Visualization of peroxisomes (microbodies) and mitochondria with diaminobenzidine. *J. Histochem. Cytochem.* 17:675–680.
24. Osumi, T., T. Tsukamoto, S. Hata, S. Yokota, S. Miura, Y. Fujiki, M. Hijikata, S. Miyazawa, and T. Hashimoto. 1991. Amino-terminal presequence of the precursor of peroxisomal 3-ketoacyl-CoA thiolase is a cleavable signal peptide for peroxisomal targeting. *Biochem. Biophys. Res. Commun.* 181:947–954.
25. Pringle, J. R., A. M. Amans, D. J. Drubin, and B. K. Haarer. 1991. Immunofluorescence methods for yeast. *In* Guide to Yeast Genetics and Molecular Biology, C. Guthrie and G. Fink, editors. Academic Press, Inc., San Diego. 565–601.
26. Santos, M. J., S. Hoefler, A. B. Moser, H. W. Moser, and P. B. Lazarow. 1992. Peroxisome assembly mutations in humans: structural heterogeneity in Zellweger syndrome. *J. Cell Physiol.* 151:103–112.
27. Santos, M. J., T. Imanaka, H. Shio, and P. B. Lazarow. 1988. Peroxisomal integral membrane proteins in control and Zellweger fibroblasts. *J. Biol. Chem.* 263:10502–10509.
28. Santos, M. J., T. Imanaka, H. Shio, G. M. Small, and P. B. Lazarow. 1988. Peroxisomal membrane ghosts in Zellweger syndrome-aberrant organelle assembly. *Science (Washington DC).* 239:1536–1538.
29. Sherman, F., G. R. Fink, and J. B. Hicks. 1986. Laboratory Course Manual for Methods in Yeast Genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York. 186 pp.
30. Shimozawa, N., Y. Suzuki, T. Orii, A. Moser, H. W. Moser, and R. J. A. Wanders. 1993. Standardization of complementation grouping of peroxisome-deficient disorders and the second Zellweger patient with peroxisomal assembly factor-1 (PAF-1) defect. *Am. J. Hum. Genet.* 52:843–844.
31. Shimozawa, N., T. Tsukamoto, Y. Suzuki, T. Orii, Y. Shirayoshi, T. Mori, and Y. Fujiki. 1992. A human gene responsible for Zellweger syndrome that affects peroxisome assembly. *Science (Wash. DC).* 255: 1132–1134.
32. Skoneczny, M., A. Chelstowska, and J. Rytka. 1988. Study of the coinduction by fatty acids of catalase A and acyl-CoA oxidase in standard and mutant *Saccharomyces cerevisiae* strains. *Eur. J. Biochem.* 174:297–302.
33. Slot, J. W., and H. J. Geuze. 1984. Gold markers for single and double immunolabeling of ultrathin cryosections. *In* Immunolabeling for Electron Microscopy. J. M. Polark, and J. M. Vandell, editors. Elsevier Science Publishing Co., Inc., Amsterdam. 129–142.
34. Small, G. M., T. Imanaka, H. Shio, and P. B. Lazarow. 1987. Efficient association of *in vitro* translation products with purified, stable *Candida tropicalis* peroxisomes. *Mol. Cell Biol.* 7:1848–1855.
35. Small, G. M., L. J. Szabo, and P. B. Lazarow. 1988. Acyl-CoA oxidase contains two targeting sequences each of which can mediate protein import into peroxisomes. *EMBO (Eur. Mol. Biol. Organ.). J.* 7:1167–1173.
36. Stevens, B. J. 1977. Variation in number and volume of the mitochondria in yeast according to growth conditions. A study based on serial sectioning and computer graphic reconstruction. *Biol. Cellulaire.* 28:37–56.
37. Suzuki, Y., N. Shimozawa, S. Yajima, T. Orii, S. Yokota, Y. Tashiro, T. Osumi, and T. Hashimoto. 1992. Different intracellular localization of peroxisomal proteins in fibroblasts from patients with aberrant peroxisome assembly. *Cell Struct. Funct.* 17:1–8.
38. Swinkels, B. W., S. J. Gould, A. G. Bodnar, R. A. Rachubinski, and S. Subramani. 1991. A novel, cleavable peroxisomal targeting signal at the amino-terminus of the rat 3-ketoacyl-CoA thiolase. *EMBO (Eur. Mol. Biol. Organ.) J.* 10:3255–3262.
39. Thieringer, R., H. Shio, Y. Han, G. Cohen, and P. B. Lazarow. 1991. Peroxisomes in *Saccharomyces cerevisiae*: immunofluorescence analysis and import of catalase A into isolated peroxisomes. *Mol. Cell Biol.* 11:510–522.
40. Tsukamoto, T., S. Yokota, and Y. Fujiki. 1990. Isolation and characterization of Chinese hamster ovary cell mutants defective in assembly of peroxisomes. *J. Cell Biol.* 110:651–660.
41. Van den Bosch, H., R. B. H. Schutgens, R. J. A. Wanders, and J. M. Tager. 1992. Biochemistry of peroxisomes. *Annu. Rev. Biochem.* 61: 157–197.
42. Van Der Leij, I., M. Van Den Berg, R. Boot, M. Franse, B. Distel, and H. F. Tabak. 1992. Isolation of peroxisome assembly mutants from *Saccharomyces cerevisiae* with different morphologies using a novel positive selection procedure. *J. Cell Biol.* 119:153–162.
43. van Dijken, J. P., M. Veenhuis, C. A. Vermeulen, and W. Harder. 1975. Cytochemical localization of catalase activity in methanol-grown *Hansenula polymorpha. Arch. Microbiol.* 105:261–267.
44. van Tuinen, E., and H. Riezman. 1987. Immunolocalization of glyceraldehyde-3-phosphate dehydrogenase, hexokinase and carboxypeptidase Y in yeast cells at ultrastructural level. *J. Histochem. Cytochem.* 35: 327–333.
45. Veenhuis, M., M. Mateblowski, W.-H. Kunau, and W. Harder. 1987 Proliferation of microbodies in *Saccharomyces cerevisiae. Yeast.* 3 77–84.
46. Wiemer, E., S. Brul, W. W. Just, M. van den Berg, P. J. Weijers, R. B. H. Schutgens, H. Van den Bosch, A Schram, R. J. A. Wanders, and J. M. Tager. 1989. Presence of peroxisomal membrane proteins in liver and fibroblasts from patients with Zellweger syndrome and related disorders: evidence for the existence of ghosts. *Eur. J. Cell Biol.* 50:407–417.
47. Wiebel, F. F., and W.-H. Kunau. 1992. The Pas2 protein essential for peroxisome biogenesis is related to ubiquitin-conjugating enzymes. *Nature (Lond.).* 359:73–78.
48. Yajima, S., Y. Suzuki, N. Shimozawa, S. Yamaguchi, T. Orii, Y. Fujiki, T. Osumi, T. Hashimoto, and H. W. Moser. 1992. Complementation study of peroxisome-deficient disorders by immunofluorescence staining and characterization of fused cells. *Hum. Genet.* 88:491–499.
49. Zhang, J. W., C. Luckey, and P. B. Lazarow. 1993. Three peroxisome protein packaging pathways suggested by selective permeabilization of yeast mutants defective in peroxisome biogenesis. *Mol. Biol. Cell.* In press.
50. Zoeller, R. A., and C. R. H. Raetz. 1986. Isolation of animal cell mutants deficient in plasmalogen biosynthesis and peroxisome assembly. *Proc. Natl. Acad. Sci. USA.* 83:5170–5174.