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

Jing Wei Zhang, Yingshi Han, and Paul B. Lazarow
Department of Cell Biology and Anatomy, Mount Sinai School of Medicine, New York 10029

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. 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-
somases, which proliferate when the yeast is grown in medium containing fatty acids (32, 45). H₂O₂ is a byproduct of this metabolism, one H₂O₂ being produced each time a fatty acid is shortened by two carbons. H₂O₂ is very toxic if it accumulates in the cell. It is mainly degraded by two isoforms 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 H₂O₂ 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 H₂O₂ when grown in the presence of oleic acid. In the absence of H₂O₂ cytotoxicity, such mutants would be expected to grow normally, provided that an alternate 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.

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 ofYPD 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 µg/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 fluorescence-linked secondary antibody (500-fold dilution) was applied to each well and incubated at room temperature for 2 h. The secondary antibody was detected with a rhodamine-conjugated secondary antibody (1:200 dilution) and mounted with chloroform/methanol/2% gelatin. The slides were observed under a Zeiss Axioshot microscope.

Table I. Strategy to Isolate Peroxisome Biogenesis (Peb) Mutants Based on the Toxicity of H₂O₂

| Yeast strains | Growth medium: carbon source | Fatty acid oxidation and production of H₂O₂ | Catalase activity and decomposition of H₂O₂ | Accumulation of H₂O₂ | Expected growth |
|---------------|------------------------------|-------------------------------------------|-------------------------------------------|---------------------|----------------|
| Wild type     | Glycerol                     | +                                         | +                                         | −                    | Normal colonies |
| Catalase-deficient | Glycerol and oleate           | −                                         | −                                         | −                    | Tiny or no colonies |
| Peb mutant    | Glycerol                     | +                                         | −                                         | +                    | Normal colonies |

Table II. Yeast Strains Used in This Study

| Name          | Genotype          | Source  |
|---------------|-------------------|---------|
| GC1-8B        | MATα, leu2-3,112, ura3-1, trp1-1, att1-1, att1-2 | (3)     |
| DCT1-2C       | MATα, leu1, arg4, att1-1 | This study |
| JW68-3A       | MATα, ura3-1, trp1-1, arg4, att1-1 | This study |
| m6-D1         | MATα, peb1-1, ura3-1, trp1-1, att1-1 | This study |
| m11-A1        | MATα, peb2-1, ura3-1, trp1-1, arg4, att1-1 | This study |
| m33-C2        | MATα, peb3-1, ura3-1, trp1-1, arg4, att1-1 | This study |
| 2m1-A4        | MATα, peb4-1, leu2-3,112, ura3-1, trp1-1, arg4, att1-1 | This study |
| m34-A4        | MATα, peb5-1, leu1, trp1-1, arg4, att1-1 | This study |
| m24-C4        | MATα, peb2-1, trp1-1, ura3-1, att1-1 | This study |
| BQS20         | MATα, ura3-1, leu2::HIS3, pol1::URA3 | This study |

Figure 1. Protection against H₂O₂ toxicity in S. cerevisiae. H₂O₂ is formed in peroxisomes when a double bond is produced in a fatty acid undergoing β-oxidative conversion to acetyl-CoA. The H₂O₂ is normally decomposed within the peroxisome by catalase isozyme A. Any H₂O₂ escaping from the peroxisome will be degraded by the cytosolic catalase isozyme T before it can damage other macromolecules, such as DNA.
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 x 10^8 ml, and grown exponentially for 16 to 18 h to a final density of approximately 5 x 10^7 cells/ml. The cells were then inoculated into YPGO medium at a density of 5 x 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) (2 mg/ml) as described by van Dijken et al. (43). After the DAB reaction, cells were fixed with vanadium permanganate. The cells were then stained with 1.5% uranyl acetate and processed as described above. In control experiments, 50 mM 3-aminobenzamide, 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 NH4Cl. Small pellets were dehydrated in ethanol at -20°C, embedded in Lowicryl K4M, and polymerized at -20°C under UV light for 1 to 2 h. Silver sections were then mounted on formvar-coated 200-mesh nickel grids and block-stained 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 proximal 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-a-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 Immunochimicals (St. Louis, MO). Zymolyase 100-T was from ICN Biomedicals, Inc. (Costa Mesa, CA).

Results

Toxicity of H2O2 Produced during Peroxisomal Olate Metabolism in Catalase-lacking Yeast

We tested the hypothesis that H2O2 produced by the peroxisomal metabolism of fatty acids would be toxic to cells lacking the protection of catalase. Two related yeast strains, GCl-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 H2O2 formation is indeed toxic to cells lacking catalase.

Isolation of Peroxisome Biogenesis 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
control, mAbs against tubulin were included (Fig. 3 B and D). 11 clones with reproducible and interesting mutant immu-
nofluorescence 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 ex-
pected to require intact peroxisomes. The mutants with al-
tered intracellular distributions of thiolase (as assessed by
fluorescence) did not grow at all (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 oleic acid 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 fur-
ther analyzed by immunofluorescence and all showed 2:2
segregation for punctate, wild-type thiolase immunofluores-
cence. A typical result is illustrated in Fig. 4: two of the four

**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 immunofluores-
cence 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 (peb1, 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 immu-
no labeling, as described below.

In one new complementation group, peb5, most cells con-
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 ml1-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 2ml, 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 tetads 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.
**Mutant Phenotypes and Complementation**

| Mutant | Thiolase immunolocalization pattern | Growth on YNO | Pebl complementation group* |
|--------|------------------------------------|---------------|----------------------------|
| m6     | Cytosolic                          | –             | 1                          |
| m13    | Cytosolic                          | –             | 1                          |
| m24    | Cytosolic                          | –             | 1                          |
| m28    | Cytosolic                          | –             | 2                          |
| 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.

Table IV. Mutant Phenotypes and Complementation

(Fig. 7). The cytoplasm of this mutant consistently appeared abnormal, with large vacuoles and lipid 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 thiase, are mislocalized to the cytosol. In each mating, the resulting diploids showed wild-type punctate thiase fluorescence. Therefore, peb4 belongs to a different complementation group than pasl-6. On the other hand, mutant m11 (peb2) failed to complement pasl, suggesting that m11 is an allele of pasl.

**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-thiase, 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 thiase into peroxi-

...omes, shown by the immunofluorescence data, was tested further by immunogold labeling. As shown in Fig. 9, wild-type yeast peroxisomes, reacted with anti-thiase, 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 peroxi-

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

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

**peb5.** In mutant m34, peb5, thiase 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 pebl, these peroxisomes were consistently observed in clusters (Fig. 6). Immunoelectron microscopy of these peroxisomes demonstrated that they contain thiase (Fig. 9). Therefore, the large fluorescent particles seen in Fig. 5 are in fact clusters of normal-sized peroxi-

...omes. 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 vacular 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 mt-

...ochondrial 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 pebl-1 and peb5-1 mutations. Growth was in YPGO for 18 h. Bar, 0.4 μm.
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-
cytosolic catalase probably reduces the amount of externally added H₂O₂ 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 immunoelectron 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, pas1-6, 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 utilized 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 H₂O₂ 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.

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

Labeled 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. Peb1 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
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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