

High-Level Expression and Molecular Cloning of Genes Encoding *Candida tropicalis* Peroxisomal Proteins

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The development of peroxisomes in the cells of *Candida tropicalis* grown on oleic acid was accompanied by a markedly high expression of peroxisomal proteins. On the basis of this finding, the nuclear DNA library of this yeast was screened by differential hybridization, and 102 clones of oleic acid-inducible sequences were isolated. Seven coding regions were found to form clusters in three stretches of the genomic DNA. Five of the regions were identified as genes for peroxisomal polypeptides (PXPs). The coding sequence for PXP-2 hybrid selected an additional mRNA for PXP-4, the subunit of long-chain acyl coenzyme A oxidase, which was the most abundant PXP. PXP-2 and PXP-4 were close in apparent molecular weight and generated similar peptides when digested with a protease. The gene for PXP-4 was adjacent to that for PXP-2 on the genome and also hybridized to the mRNA coding for PXP-5. These and other similar results suggest that the genes for the peroxisomal proteins of this organism arose by duplication of a few ancestral genes.

The enzyme composition of peroxisomes, intracellular compartments generating and degrading hydrogen peroxide, varies widely in contrast to their ubiquitous distribution. The peroxisomes of methanol-assimilating yeasts contain only a few enzymes (10, 40), whereas the equivalents of fatty acid-utilizing yeasts have, as in the case of hepatic peroxisomes, more than a dozen enzymes in addition to a β -oxidation system for long-chain fatty acids (1, 6, 12, 20, 28, 39). In yeast cells, unlike in hepatocytes, fatty acids are oxidized solely in peroxisomes (20), which have a novel long-chain acyl coenzyme A synthetase whose product is exclusively utilized for β -oxidation but not for lipid synthesis (17, 18, 28).

Candida tropicalis pK233 is a typical yeast strain which assimilates fatty acids or alkanes as the sole source of carbon and energy. The cells grown on glucose contain few peroxisomes, whereas those grown on oleic acid or *n*-alkanes (C₁₀-C₁₃) develop large numbers of the organelles (30). The peroxisomes of this yeast strain have been purified to near homogeneity (16), and the induction of many associated enzymes has been reported (39). *C. tropicalis* is thus a suitable unicellular system for studying the biogenesis of peroxisomes and its regulation. In this regard, special attention was directed to reports (29, 31) suggesting the presence of DNA in the peroxisomes of this organism. Extensive searches for the proposed DNA, however, provided no evidence for this and led us to conclude that the biogenesis of peroxisomes must be entirely controlled by nuclear genes (16).

We report here that the development of peroxisomes in cells grown on oleic acid is accompanied by a markedly high expression of peroxisomal proteins. On the basis of this finding, we isolated oleic acid-inducible DNA sequences by differential hybridization. At least five oleate-inducible sequences were identified as genes encoding peroxisomal polypeptides (PXPs) and were found to form clusters in the genomic DNA fragments. Genes for the peroxisomal proteins of this organism may have arisen by duplication of a few ancestral genes.

MATERIALS AND METHODS

Enzymes. Egg white lysozyme, rabbit muscle creatine phosphokinase, micrococcal nuclease, and papain were obtained from Sigma Chemical Co. Calf intestine alkaline phosphatase was purchased from Boehringer-Mannheim Biochemicals. T4 DNA ligase was purchased from Takara Shuzo Inc.; staphylococcal V8 protease was purchased from Miles Laboratories Inc.; and AMV reverse transcriptase was purchased from Seikagaku Kogyo Inc. Zymolyase 60000 was a gift of Kirin Brewery Co. The source of other enzymes has been previously described (16).

Yeast cells and peroxisomes. Cells of *C. tropicalis* pK233 (ATCC 20336) were grown at 30°C in YPBO medium containing 1% oleic acid or in YPBG medium containing 2% glucose as previously described (16). Peroxisomes were purified by the method of Kamiryo et al. (16), using sucrose density gradient centrifugation. Radioactive peroxisomes, as the reference for PXPs, were prepared from cells grown in basal medium (15) supplemented with 1% oleic acid, 1 mg each of 19 common amino acids except leucine per ml, and 4 μ Ci of L-[4,5-³H]leucine (65 Ci/mmol; Amersham Corp.) per ml.

General methods. The following procedures were carried out by standard methods described by Maniatis et al. (25) unless otherwise indicated: isolation of the total cellular RNA with guanidium-cesium chloride, selection of polyadenylated [poly(A)⁺] RNA, digesting DNA with restriction endonuclease, agarose gel electrophoresis of DNA in Tris-acetate buffer and of glyoxalated RNA, bacterial transformation with CaCl₂, large-scale as well as rapid small-scale isolation of plasmid DNA by alkaline lysis, transfer and hybridization of DNA on nitrocellulose filter, constructing maps of restriction sites, autoradiography, and hybrid selection of specific mRNA. Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis was conducted by Laemmli (21). Protein was determined by the method of Lowry et al. (24).

Cell-free translation of yeast mRNA. To isolate total cellular RNA, ca. 10 g (wet weight) of cells was suspended in 40 ml of a solution consisting of 5 M guanidium thiocyanate (Fluka), 30 mM sodium citrate (pH 7.0), 0.5% Sarkosyl, 0.1 M 2-mercaptoethanol, and a few drops of Antifoam (Na-

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karai) and were disrupted with glass beads (3-g beads with a diameter of 0.45 to 0.50 mm per g of wet cells) in a cell homogenizer (Braun) at high speed for 1 min without cooling. The supernatant freed from the glass beads by low-speed centrifugation was layered onto a solution (one-half volume of the sample) containing 5.7 M CsCl, 0.1 M EDTA (pH 7.0), and 0.2% diethylpyrocarbonate, followed by the standard method. Poly(A)⁺ RNA was selected by two cycles of oligodeoxythymidylate-cellulose (Miles) chromatography. Cell-free translation with micrococcal nuclease-treated rabbit reticulocyte lysate (32) was carried out with some modification (14). The translation products labeled with L-[³⁵S]methionine (Amersham) were subjected to SDS-polyacrylamide gel electrophoresis and detected by fluorography with sodium salicylate (2).

Preparation of nuclear DNA. The nuclei of glucose-grown cells were purified to free the library of mitochondrial DNA. Thirty grams (wet weight) of cells washed with 20 mM Na₂SO₃ was suspended in 120 ml of 0.5 M KCl containing 10 mM Na₂SO₃ and 20 mM 3-(*N*-morpholino)propanesulfonate (MOPS) (pH 6.5) and was converted to spheroplasts by incubation with 45 mg of Zymolyase 60000 at 30°C for 30 min. The resulting spheroplasts were homogenized in 90 ml of 18% Ficoll 400 (Pharmacia Fine Chemicals, Inc.) in 20 mM MOPS (pH 6.5)–3 mM CaCl₂ with a Teflon-glass homogenizer by five “down-and-up” strokes. The homogenate was centrifuged at 1,600 × *g* for 10 min. After decanting the clear supernatant, a slimy but not tightly packed material was collected (ca. 60 ml). About 15 ml of this material was applied to a gradient consisting of 20 ml of 2.5 M sorbitol–30% (wt/vol) glycerol and 5 ml of 1 M sorbitol–15% glycerol, both solutions containing 9% Ficoll 400, 20 mM MOPS (pH 6.5), and 3 mM CaCl₂. The gradient was centrifuged at 15,000 × *g* for 30 min, and the nuclei which sedimented to the bottom of the tube were collected with the aid of 20 ml of 20 mM MOPS (pH 6.5)–3 mM CaCl₂ and precipitated. The precipitate was thoroughly suspended in 20 ml of 0.2 M NaCl. The suspension was made 0.15 M NaCl–0.1 M EDTA, and high-molecular-weight nuclear DNA was prepared by the method of Cryer et al. (5).

Construction and screening of genomic DNA library. The nuclear DNA was partially digested with *Sau*3AI, and size-fractionated 10- to 20-kilobase-pairs (kb) fragments were ligated to *Bam*HI-cleaved pBR322 digested to completion with alkaline phosphatase. The ligated DNA was introduced into *Escherichia coli* HB101 via transformation. Replica filters (Millipore Corp.; HATF) of transformants at high colony density (11) were screened by differential hybridization (38). Because of the low guanine-cytosine content (35%) (26, 37), hybridization was carried out at 62°C for 20 to 45 h in a solution containing 5× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), 10× Denhardt solution, 0.1 mg of denatured salmon sperm DNA per ml, 0.1% SDS, and 1 × 10⁶ to 2 × 10⁶ cpm of the probe per ml. Southern blot hybridization (36) was performed under the same conditions. A single-stranded cDNA probe was prepared with [α-³²P]dCTP (200 to 300 Ci/mmol; Amersham) as described by St. John and Davis (38).

Hybrid selection and peptide mapping. DNA of the subcloned plasmid was immobilized on nitrocellulose (Schleicher & Schuell, Inc.; BA85) by the standard method. The total cellular RNA from oleate-grown cells was hybridized (at 40°C overnight and washed at 50°C) to and eluted from the filter by the procedure of Ricciardi et al. (33). Cell-free translation was carried out as described above. The translation product synthesized with the selected mRNA was

mixed with the authentic PXP and digested partially with staphylococcal V8 protease or papain in gel slices by the method of Cleveland et al. (4).

Hybrid-arrested cell-free translation. Poly(A)⁺ RNA from oleate-grown cells was hybridized with a restriction fragment or subcloned plasmid DNA and translated in the nuclease-treated reticulocyte lysate by the procedure of Flavell et al. (8).

RESULTS

Expression of peroxisomal proteins. The total proteins of *C. tropicalis* cells grown on oleic acid and on glucose were compared by SDS-polyacrylamide gel electrophoresis (Fig. 1). Lane 1 represents the polypeptides of highly purified peroxisomes distinct from those of mitochondria (lane 7). PXP-4 is, for example, the subunit of long-chain acyl coenzyme A oxidase, and PXP-9 is that of catalase (unpublished data). Lane 2 is the polypeptide pattern of the homogenate from oleate-grown cells; most of the predominant bands of more than 52 kilodaltons (kd) were associated with peroxisomes. Densitometric analysis indicated that PXP-4, the most abundant PXP, accounted for more than 3% of total cellular proteins. On the contrary, the homogenate from glucose-grown cells contained mitochondrial proteins but no PXP (lane 6). PXPs were detected by 2 h after transferring the cells to the oleate medium (lanes 3 to 5); during this period, the cells underwent little growth. These findings suggest that the development of peroxisomes was accompanied by a de novo synthesis of individual peroxisomal proteins.

The levels of mRNA coding for PXPs were next examined with an mRNA-dependent cell-free translation system. Many dominant translation products of oleate mRNA, poly(A)⁺ RNA from oleate-grown cells (Fig. 2, lane 2), had

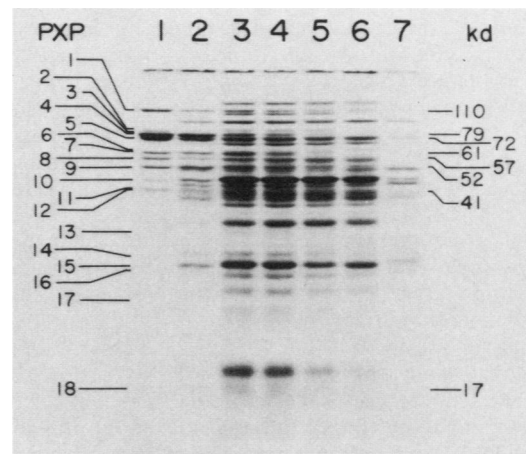


FIG. 1. PXPs and polypeptides in cells grown on oleic acid or glucose. Polypeptides in purified organelles and whole cell homogenates were separated on a 10% polyacrylamide gel and stained with Coomassie brilliant blue. Cell homogenates were prepared as described by Kamiryo and Numa (19) in the presence of 0.1 mg phenylmethylsulfonyl fluoride per ml. Lane 1, Purified peroxisomes; lane 2, homogenate from cells grown in YPBO overnight; lanes 3 to 5, those from cells grown in YPBG overnight, washed, and incubated in YPBO for 3 h (lane 3), 2 h (lane 4), or 1 h (lane 5); lane 6, that from cells grown in YPBG overnight; and lane 7, mitochondria. The numbering of PXP (left) and apparent molecular weight in kilodaltons (right) are indicated. It is uncertain whether PXP-3 and PXP-7 are independent entities or the precursors or apoproteins of other PXPs.

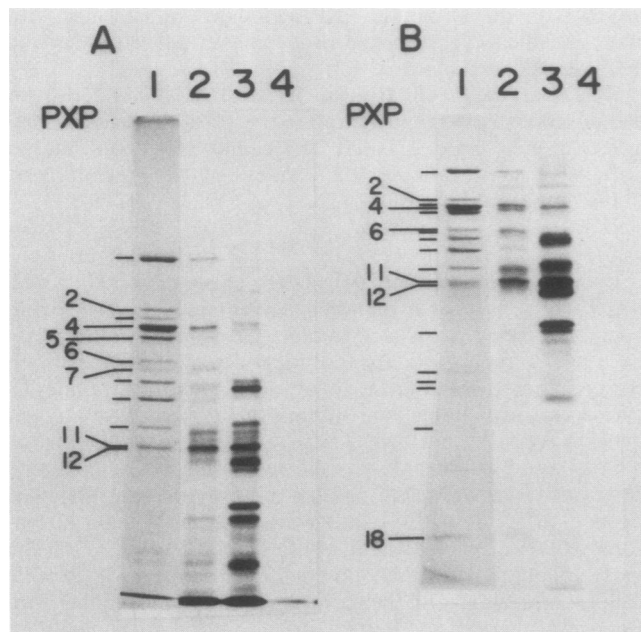


FIG. 2. Cell-free translation of mRNA isolated from cells grown on oleic acid or glucose. About 0.4 μg of poly(A)⁺ RNA was translated in 20 μl of the nuclease-treated reticulocyte lysate system. The resulting translation products in 0.5 μl of the mixture were diluted with 4.5 μl of 50 μg of polymethylsulfonil fluoride per ml, separated on 7% (A) and 10% (B) polyacrylamide gels, and detected by fluorography. Lane 1, PXP detected by staining; lane 2, translation products of oleate mRNA; lane 3, those of glucose mRNA; and lane 4, those without exogenous RNA.

essentially the same mobility as did PXP (Fig. 2, lane 1). Since few of these polypeptides could be found in the products of glucose mRNA (lane 3), many PXP were readily correlated to the translation products in lane 2. This observation suggests that most PXP are synthesized at the mature size and is consistent with reports that hepatic (23) and yeast (34, 41) peroxisomal enzymes translated in vitro have the same molecular weights as the respective mature enzymes. The high-level expression of genes encoding peroxisomal proteins prompted us to isolate the genes by screening for oleate-inducible DNA sequences. Differential hybridization should exclude constitutive genes such as those for mitochondrial proteins.

Cloning of oleate-inducible DNA sequences. A DNA library, constructed with nuclear DNA from *C. tropicalis* and pBR322, was screened for oleate-inducible sequences. Colonies giving positive hybridization signals when probed with oleate cDNA, single-stranded cDNA prepared from oleate mRNA, but not when probed with glucose cDNA were picked as presumptive oleate-inducible sequences. All clones scored as presumptively inducible were twice re-screened, since on some occasions, the colony filter hybridization provided false signals. Of ca. 20,000 clones screened in this manner, 102 were clearly inducible.

Because of the large size of the library screened, a certain sequence was expected in several different clones. Therefore, the plasmid DNAs were isolated from individual clones and analyzed so as to group them by Southern blot hybridization. Three independent groups were selected for further study; each group contained at least four different plasmids, all quite likely to possess genes encoding PXP, since the

restriction fragments they generated gave particularly strong signals to oleate cDNA but were clearly negative to glucose cDNA. Only fragments corresponding to *OLE1* (see below) gave weak signals to oleate cDNA.

Cluster of oleate-inducible genes. Locating the transcriptional coding region is necessary for identification of the protein product of an oleate-inducible gene. Figure 3 shows seven coding regions in genomic DNA cloned in three independent plasmids. A restriction map of the DNA stretch was confirmed by maps of several plasmids in a group. No evidence has yet been found for the linkage between the three DNA stretches. Unexpectedly, each stretch contained more than one coding region, suggesting clusters of genes for peroxisomal proteins. In fact, five regions were identified as genes for PXP. The coding regions were designated as shown in Fig. 3 on the basis of the results of the experiments described below (Fig. 4 and 5).

Figure 4 shows the cell-free translation products of mRNA selected by hybridization with a subcloned DNA containing a single coding region. Six of the regions were correlated to six PXP by reference to the translation products of the total oleate mRNA, i.e., *POX2* to PXP-2 (lane 2), *POX4* to PXP-4 (lane 3), *POX6* to PXP-6 (lane 4), *POX11* to PXP-11 (lane 6), *OLE2* to PXP-12 (lanes 7 and 10), and *POX18* to PXP-18 (lane 8). The mRNA selected by *POX4*, *POX11*, or *OLE2* translated an additional PXP-like polypeptide. The sequence of *POX2* selected two additional mRNAs for 72- and 69-kD polypeptides. Interestingly, the additional polypeptides have apparent molecular weights very close to those of the respective primary PXP. No translation product was detected in the experiment on *OLE1* (lane 9). The assignment based on the hybrid selection experiment is tentative and requires further evidence for the identity of the translation products.

Identification of the product translated from hybrid-selected mRNA. The labeled cell-free translation product of a hybrid-selected mRNA was mixed with the tentatively assigned

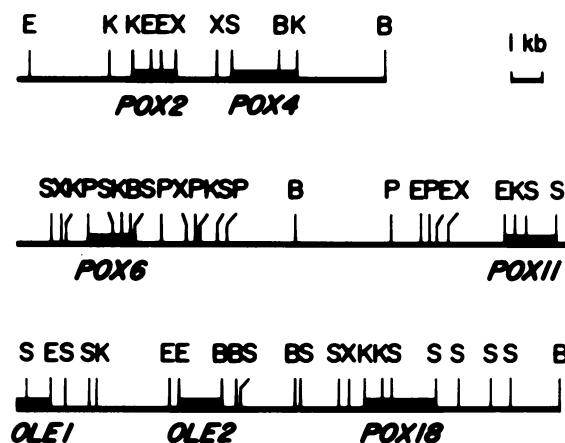


FIG. 3. Linkage of oleate-inducible coding regions. The restriction maps of three genomic sequences cloned into representative plasmids (from top, pC1, pC5, and pC7) are indicated. Restriction endonuclease sites are abbreviated as follows: B, *Bam*HI; E, *Eco*RI; K, *Kpn*I; P, *Pst*I; S, *Sal*I; X, *Xho*I. Transcriptional coding regions (thick bars) were located by Southern analysis with an oleate cDNA probe. Each region indicates the restriction fragment giving a strong signal and does not necessarily include the entire transcriptional unit. The region assigned to a PXP was designated as *POX*, followed by the number of the PXP. The region not correlated to PXP was temporarily termed *OLE*.

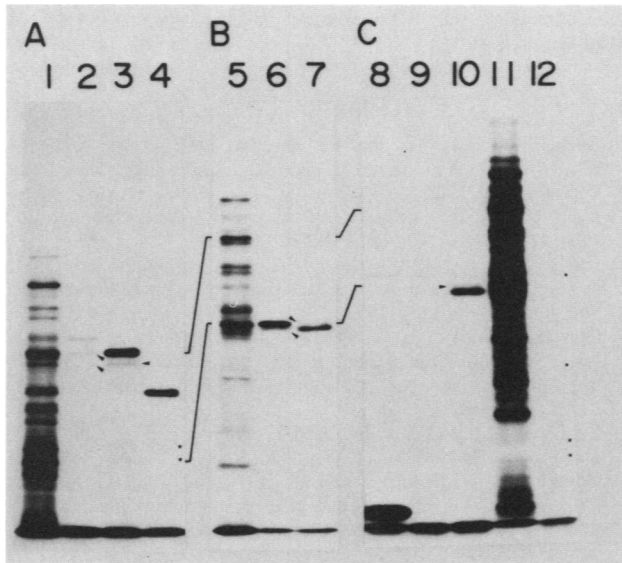


FIG. 4. Cell-free translation of mRNA hybrid selected with oleate-inducible sequence. The respective coding region was subcloned either by ligation of a suitable restriction fragment to pBR322 or by elimination of needless or interfering fragments from the original plasmid. About 50 μ g of the plasmid DNA was immobilized onto three filters of 3-mm squares and hybridized with 100 to 300 μ g of the total cellular RNA from oleate-grown cells. The RNA eluted was added to 20 μ l of a nuclease-treated reticulocyte lysate system. The translation products in 1 μ l of the mixture were separated on a 7% (A), 9% (B), or 10% (C) polyacrylamide gel. Lanes 1, 5, and 11, Translation products of the total oleate mRNA; lane 2, product of mRNA selected by *POX2*; lane 3, that selected by *POX4*; lane 4, that selected by *POX6*; lane 6, that selected by *POX11*; lanes 7 and 10, that selected by *OLE2*; lane 8, that selected by *POX18*; lane 9, that selected by *OLE1*; and lane 12, translation products without exogenous RNA. Additional translation products are marked with arrowheads. Dots indicate products due to endogenous mRNA.

authentic PXP and digested partially with staphylococcal V8 protease or papain in various amounts. The proteolytic peptide map of translation product was detected by fluorography (Fig. 5A), and that of PXP was visualized by staining (Fig. 5B). A comparison of the two peptide maps indicated that the product translated from the mRNA selected by *POX2* was identical to authentic PXP-2 (lanes 1 to 3). This identity was also confirmed for the products of mRNA selected by *POX4* (lane 7), *POX6* (lane 9), *POX11* (lane 10), and *POX18* (lane 12). The additional translation products observed for *POX2* (72 kd) and *POX4* were likewise identified as PXP-4 (lanes 4 to 6) and PXP-5 (lane 8), respectively. We emphasize here the resemblance between the peptide maps of PXP-2 and PXP-4 (Fig. 5B). Peptides generated from the translation product related to *OLE2* agreed with minor peptides from PXP-12 but disagreed with dominant ones (lane 11).

Four coding regions were subjected to hybrid-arrested cell-free translation for further confirmation. The results (data not shown) were consistent with previous assignments. The translation of a major product correlated to PXP-4, PXP-6, and PXP-18 was apparently inhibited by hybridization of the total oleate mRNA with *POX4*, *POX6*, and *POX18*, respectively, whereas that of the product assigned to PXP-11 was partially inhibited with *POX11*. The translation product (61 kd) of the mRNA selected by *POX6* was distinct from PXP-6 (62 kd) and rather close to PXP-7 (61 kd)

in apparent molecular weight. Nevertheless, we compared the peptide map of this product with that of PXP-6 which was more abundant than PXP-7. The reason was that the 61-kd translation product was oleate inducible and an abundant single entity as evidenced by hybrid-arrested cell-free translation, where the sequence of *POX6* inhibited the translation of the 61-kd product completely. The peptide map of this product was compatible with that of PXP-6 (Fig. 5) but not with that of PXP-7 nor PXP-8 (data not shown).

DISCUSSION

Peroxisomes consist of many enzymes not necessarily related to each other in metabolism. This implies that the regulatory mechanism for the biogenesis of this organelle must differ from the specific control of a single metabolic pathway or even the general control of several related pathways, such as general amino acid control (7, 13). To study such a complex mechanism of regulation, we focused our attention on the proteins expressed at a high level when induced, aside from metabolic consideration.

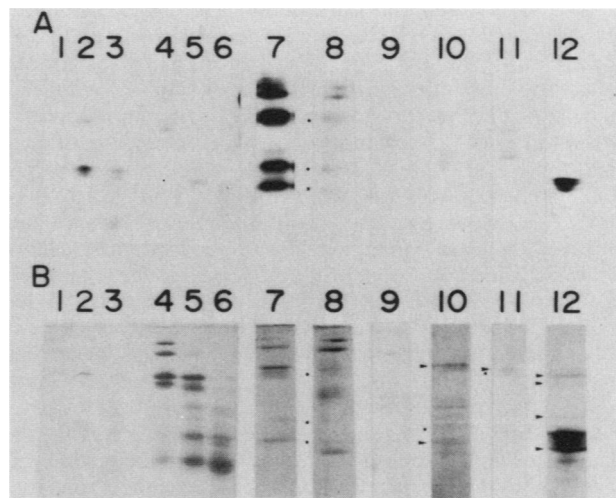


FIG. 5. Peptide mapping of the cell-free product translated from hybrid-selected mRNA and authentic PXP. Hybrid-selected mRNA was translated as was the experiment shown in Fig. 4. The translation product in 1 to 5 μ l of the lysate system was mixed with 100 to 500 μ g of purified unlabeled peroxisomes and subjected to SDS gel electrophoresis on the polyacrylamide of an appropriate concentration. After brief staining, the band of the PXP in question was cut out, applied to a second slab gel (15% polyacrylamide), and digested during the subsequent electrophoresis with at least three different amounts (5 ng to 10 μ g) of staphylococcal V8 protease. The peptides formed were detected by fluorography for the translation product (A) and by staining for the authentic PXP (B). Lanes 1 to 3, Major translation product selected by *POX2* and authentic PXP-2, using increasing amounts of protease; lanes 4 to 6, minor product selected by *POX2* (72 kd) and PXP-4; lane 7, major product selected by *POX4* and PXP-4; lane 8, minor product selected by *POX4* and PXP-5; lane 9, product selected by *POX6* and PXP-6; lane 10, major product selected by *POX11* and PXP-11; lane 11, major product selected by *OLE2* and PXP-12; and lane 12, product selected by *POX18* and PXP-18. PXP-6, PXP-7, and PXP-8 were electrophoretically separated before mixing with the translation product selected by *POX6*. No translation product was included in lanes 9 to 11 of panel B to avoid proteins from the reticulocyte lysate; the extent of proteolysis was nearly the same to the corresponding lanes of panel A. Dots indicate peptides due to contamination of the major product and the primary or nearby PXP, and arrowheads denote protease and its autolytic products.

We found a markedly high expression of peroxisomal proteins in the oleate-grown cells of *C. tropicalis* and used differential hybridization to isolate genes expressed specifically in these cells. The collection of 102 clones would be expected to include all of the oleate-inducible genes, since the size of the genomic library screened was ca. three times that necessary to contain a particular sequence at 99% probability. For calculation purposes (3), the total genome of *C. tropicalis* was assumed to be ca. 15,000 kb. This expectancy was supported by the fact that each of the three DNA stretches containing clearly inducible genes was cloned in at least four different plasmids. Of the seven coding regions, five were actually assigned to PXP.

Southern analysis showed coding regions to exist in clusters. The distances between two regions (ca. 2 to 12 kb) agreed well with those found in other eucaryotic gene clusters, e.g., clusters of human α -like (22) and β -like (9) globin genes and yeast acid phosphatase genes (27, 35). These gene clusters were thought to arise by duplication of ancestral genes, since the genes in a cluster showed a high degree of functional and structural homology. The cluster of PXP genes may also result from gene duplication.

It should be noted that some coding sequences hybridized with more than one mRNA species, i.e., mRNAs coding for the primary PXP and for one or two additional polypeptides. The additional minor translation product was very similar in electrophoretic mobility to other PXP. The minor products related to *POX2* (72 kd) and *POX4* were indeed identified as PXP-4 and PXP-5, respectively; those related to *POX11*, *OLE2*, and *POX2* (69 kd) were probably PXP-12, PXP-11, and PXP-5, respectively, although their identity has not been confirmed by peptide mapping. It is of particular interest that the primary and additional products were similar in apparent molecular weight. It is unlikely that the additional PXP-like product emerged by degradation of the primary PXP or its mRNA, since the minor product for *OLE2* was larger than the primary product. The presence of the respective genes excludes the possibility that the two polypeptides were produced by an alternative expression of a single gene. The selection of nonspecific mRNA was also unlikely; thus, we speculated that the genes for PXP arose by gene duplication and the sequence homology permitted selection of the counterpart under a rather relaxed condition of hybridization, although it was alternatively possible that the minor mRNA was hybrid selected by a putative repetitive sequence. The resemblance between the peptide maps of PXP-2 and PXP-4 provides strong support for homology in the translatable region of the two genes. *POX2*, *POX4*, and the gene for PXP-5 may constitute a multigene family, and perhaps *POX11* and the gene for PXP-12 or *OLE2* constitute another. The low abundance of PXP-2 possibly rendered *POX4* incapable of selecting the mRNA for PXP-2. The gene duplication may explain why *C. tropicalis* has more peroxisomal enzymes than methylotropic yeast and why many hydrogen peroxide-producing oxidases are localized in the peroxisomes.

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