Cloning of cDNA coding for peroxisomal acyl-CoA oxidase from the yeast Candida tropicalis pK233

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ABSTRACT Candida tropicalis pK233 cells were grown with n-alkanes as carbon source to induce the synthesis of peroxisomal proteins and the proliferation of peroxisomes. Poly(A)* RNA was isolated and used to construct a cDNA library by insertion of double-stranded reverse transcripts into the Pst I site of pBR322 followed by cloning in Escherichia coli. Clones complementary to mRNAs induced by growth on alkanes were selected by differential DNA dot-blot analysis using [32P]cDNA reverse-transcribed from poly(A)* RNA of glucose-grown cells (which contain few peroxisomes) or of alkanegrown cells. Among these clones, one containing a 1.7-kilobase insert coding for acyl-CoA oxidase (the first enzyme in the peroxisomal β-oxidation pathway) was identified by hybridization-selection translation and immunoprecipitation. By RNA blot analysis, the acyl-CoA oxidase mRNA was estimated to be ~2.2 kilobases long, of which 3.1 kilobases are required to code for the ~76-kDa protein. Since the mRNA is polyadenylated, there appears to be little additional nontranslated region. Cell-free mRNA translation and RNA dot-blot hybridization analyses demonstrated that, whereas glucose-grown C. tropicalis contained little or no acyl-CoA oxidase mRNA, alkanegrown cells contained so much of this mRNA as to make acyl-CoA oxidase one of the major in vitro translation products.

The peroxisome is a cell organelle that is present in most, if not all, eukaryotic cells, from fungi to mammals; its abundance and metabolic capacity vary with cell type (1, 2). Among its functions, the peroxisome catalyzes the β-oxidation of fatty acids; this occurs in many cell types including rat liver (3–5) and alkanegrown yeast (6).

The biogenesis of peroxisomes has features that distinguish it from the biogenesis of other organelles. Peroxinsomal proteins are encoded by nuclear genes (7) and are synthesized on free polysomes (8–16). With one exception, thiolase (12, 15, 17), all peroxinsomal proteins studied thus far are synthesized at their mature sizes, rather than as larger precursors, in both rat liver (10–14, 17–20) and yeast (16, 21, 22). This includes a major integral membrane polypeptide (14). Rat liver apocatalase monomer is transported into the peroxisome and assembled into a tetrameric hemoprotein without any apparent post-translational modification of its primary structure (20).

How are these proteins targeted to peroxisomes? In an attempt to answer this question through an analysis of the detailed molecular structure of peroxinsomal proteins, we have undertaken the cloning of cDNAs coding for these proteins.

Candida tropicalis (Castellani) Berk (ATCC 20336) is a favorable experimental organism for this purpose. When this yeast is grown on n-alkanes (C10–C13) or oleic acid as the carbon source, peroxinsomal forms in large numbers and the activity of the peroxinsomal β-oxidation system is high (6, 23–25). In contrast, when the yeast is grown on glucose, peroxinsomes are few in number and the β-oxidation activity is low or undetectable (24, 25). Growth on alkanes or fatty acids causes a striking induction of a dozen major translatable mRNAs, many of which code for peroxinsomal proteins (26).

These facts suggest the following strategy for isolating cDNA clones coding for peroxinsomal proteins. (i) Create a library of DNAs complementary to mRNAs of alkanegrown C. tropicalis. (ii) Screen the library by differential hybridization using [32P]cDNAs complementary to glucose-grown and to alkanegrown C. tropicalis mRNA. (iii) Analyze the clones complementary to alkanegrown mRNAs by hybridization-selection translation and immunoprecipitation.

In this paper, we report the isolation by this procedure and the partial characterization of a cDNA clone coding for acyl-CoA oxidase, the first enzyme of the peroxinsomal β-oxidation system. In C. tropicalis, this oxidase is an octameric flavoprotein (27–29). Some of these results have been reported in abstract form (30).

MATERIALS AND METHODS

Cultivation of Yeast. For RNA preparations, C. tropicalis Berk strain pK233 (ATCC 20336) (31) was cultured at 30°C with shaking either for 14.5 hr in a medium containing n-alkanes (a mixture of C10–C13) or for 9.5 hr in a medium containing glucose (32).

RNA Isolation and Construction of an Alkane-Grown C. tropicalis cDNA Library. Total RNA was isolated by phenol/chloroform/isoamyl alcohol extraction (33). Poly(A)* RNA was isolated by oligo(dT)-cellulose chromatography according to Aviv and Leder (34). Double-stranded cDNA complementary to poly(A)* RNA from alkane-grown cells was synthesized according to the procedure of Wickens et al. (35) and inserted into the Pst I site of pBR322 by dG-dC tailing (36, 37). Escherichia coli RRI1 cells were transformed with the recombinant plasmid plasmid by the calcium chloride procedure of Schleifer and Wensink (38) except that the calcium medium contained 5 mM Tris·HCl, pH 7.6/5 mM MgCl2/100 mM CaCl2/250 mM KCl. Before transformation, the cells in the calcium medium supplemented with 15% (vol/vol) glycerol were rapidly frozen once in dry ice/ethanol and stored at −80°C until use (38). Transformants were selected for tetracycline resistance and ampicillin sensitivity.

Selection of the Clone Coding for Acyl-CoA Oxidase. Clones coding for mRNAs enriched by growth on alkanes were selected by differential DNA dot-blot hybridization. Minipreparations of plasmid isolated from 1.5 ml of saturated cultures (39) were denatured in 20 μl of 0.5 M NaOH and then neutralized and diluted by the successive addition of 30 μl of 1 M NaCl/50 mM Tris·HCl, pH 8.0/0.5 mM EDTA, 50 μl of 1 M Tris·HCl, pH 6.8, and 100 μl of H2O. After further dilution in the same medium, the plasmid was spotted onto

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nitrocellulose (1% of the isolate per spot) by means of a dot-blot apparatus (Schleicher & Schuell). The nitrocellulose was baked for 2 hr at 80°C in vacuo and prehybridized overnight at 65°C with 50 μg denatured salmon sperm DNA per ml in 0.6 M NaCl/0.34 M Na phosphate, pH 6.2/3 mM EDTA/1% sarcosyl/2.5% dextran sulfate/10 μg of poly(A) per ml. Hybridization was performed under the same conditions with 32P-labeled cDNA reverse-transcribed from mRNA isolated from either glucose- or alkane-grown cells. The nitrocellulose filters were washed four times at 65°C in 0.3 M NaCl/30 mM Na citrate, pH 7.0/0.1% NaDodSO4/0.1% Na pyrophosphate.

Some clones coding for alkane-enriched mRNAs were characterized further by hybridization-selection of mRNA (40) and in vitro translation in a reticulocyte-lysate system (14, 41). Immunoprecipitations were carried out (14) with antiserum against acyl-CoA oxidase (26). Proteins were analyzed by NaDodSO4/PAGE (42) and fluorography (43) for 2–5 days. Purified C. tropicalis acyl-CoA oxidase (27) was the generous gift of S. Shimizu (Kyoto, Japan).

**RBA Dot-Blot and RNA Blot Hybridizations.** RNA dot-blot hybridization was performed according to the procedure of Andrews et al. (44), except that prehybridization and hybridization were done according to Rayal et al. (45).

For RNA blot analysis, RNA was denatured and fractionated by electrophoresis in 1% formaldehyde agarose gels (46). Transfer to nitrocellulose paper, prehybridization, hybridization, and washing were performed as described by Thomas (47).

Recombinant plasmids used for hybridization to RNA were purified by alkaline lysis followed by CsCl density-gradient centrifugation (48, 49) and by Biogel A-15m (200–400 mesh) column chromatography. They were radiolabeled by nick-translation with [α-32P]dCTP as labeled deoxynucleotide (50). Autoradiograms of filter hybridizations were exposed for 0.5–3 days at −80°C between two DuPont Cronex Lightning Plus intensifying screens.

**Analysis of the Recombinant Plasmid by Restriction Endonuclease Digestion.** Plasmid minipreparations prepared by alkaline lysis (48, 49) were digested by restriction endonucleases according to specifications of the manufacturers (Bethesda Research Laboratories and New England Biolabs) in the presence of DNase-free bovine pancreatic RNase (20 μg/ml, 70 units/mg; P-L Biochemicals). Cleavage products were separated by electrophoresis in agarose gels (1%, 2%, or 2.5% agarose) containing ethidium bromide (5 μg/ml). Standards were a HindIII digest of λ phage and a Hae III digest of phage dX174 (Bethesda Research Laboratories).

**RESULTS**

**Identification of the cDNA Clone to Acyl-CoA Oxidase.** A differential DNA dot-blot analysis of 48 randomly selected clones is illustrated in Fig. 1. Arrowheads identify three clones that hybridize strongly with cDNA reverse-transcribed from mRNA of alkane-grown cells and hardly at all with cDNA similarly prepared from glucose-grown cells.

Clones selected in this way were further characterized by hybridization-selection translation, as shown in Fig. 2 for clone 1:18 (b6 of Fig. 1). The mRNA selected by the insert of this clone gave, upon cell-free translation, a single polypeptide of ~76 kDa (lane 3), which comigrated in NaDodSO4/PAGE with a major translation product of poly(A)+ RNA of alkane-grown cells (lane 2) and was not detected in the translation products of poly(A)+ RNA of glucose-grown cells (lane 1). In another experiment, this major band appeared as a doublet (Fig. 3, lane 1, dots) and the hybrid-selected translation product comigrated with the upper of the two bands (Fig. 3, lane 2).

Antiserum against acyl-CoA oxidase immunoprecipitated the hybridization-selected translation product (Fig. 2, lane 7) and a major 76-kDa translation product of poly(A)+ RNA from alkane-grown cells (lane 5) but not from glucose-grown cells (lane 4). Preimmune serum did not precipitate the 76-kDa translation product (lane 6).

**Fig. 1.** Differential DNA dot-blot hybridization analysis of randomly selected clones. Equal amounts of plasmid (20–30 ng) were spotted on duplicate nitrocellulose filters and hybridized with 2.5 × 106 cpm of 32P-labeled cDNA reverse-transcribed from poly(A)+ RNA of either alkane-grown cells (6.9 × 105 cpm/μg) (Upper) or glucose-grown cells (2.3 × 105 cpm/μg) (Lower). Arrowheads show clones coding for alkane-induced mRNAs.

**Fig. 2.** Identification of acyl-CoA oxidase as in vitro translation product of mRNA selected by hybridization to recombinant clone 1:18 (b6 in Fig. 1). Five micrograms of plasmid purified by CsCl density-gradient centrifugation was bound to nitrocellulose and hybridized with ~30 μg of poly(A)+ RNA from alkane-grown cells. After washing, the specifically hybridized mRNA was eluted and translated in vitro. Fluorogram of NaDodSO4 gel. Lanes 1–3, total translation products of poly(A)+ RNA of cells grown in glucose medium (G) or in alkane medium (A) and of hybridization-selected poly(A)+ RNA from alkane-grown cells (HS-A). Lanes 4, 5, and 7, immunoprecipitates with antiserum against acyl-CoA oxidase from a 10-fold greater volume of translation mixtures G, A, and HS-A. Lane 6, immunoprecipitation with preimmune serum from translation mixture A. STD, molecular mass standards.
was estimated by RNA blot analysis with nick-translated clone 1:18 as the hybridization probe. As seen in Fig. 5, a single RNA species from poly(A)$^+$ RNA of alkane-grown cells hybridized to the probe. This RNA is ≈2.2 kilobases long. Accordingly, the cDNA insert of 1.7 kilobase pairs of clone 1:18 codes for ≈77% of the complete mRNA.

An attempt was made to quantitate the increase in mRNA coding for acyl-CoA oxidase in alkane-grown cells as compared to glucose-grown cells by RNA dot-blot hybridization with nick-translated clone 1:18 as the hybridization probe (Fig. 6). Poly(A)$^+$ RNA from alkane-grown cells gave an intense signal (lane c), whereas hybridization to poly(A)$^+$ RNA from glucose-grown cells was so low (lane b) that it could only be detected at longer exposures (not shown). The radioactivity in the individual spots was quantitated by liquid scintillation counting, but the amount of radioactivity in the glucose spots was too low for accurate determination.

**DISCUSSION**

In this paper, we report the identification of a cDNA clone complementary to an mRNA coding for the peroxisomal acyl-CoA oxidase of the yeast *C. tropicalis*. The results demonstrate the success of the strategy of first selecting from a cDNA library those clones that are complementary to mRNAs induced by growth on alkanes (Fig. 1). The acyl-CoA oxidase clone was identified by its hybridization selection of an mRNA that codes for a polypeptide that is immunoprecipitated by antiserum to acyl-CoA oxidase (Figs. 2 and 3).

From the size of the acyl-CoA oxidase translation product estimated by NaDodSO4/PAGE (≈76 kDa), it can be calculated that the coding region of the mRNA for this protein is ≈2.1 kilobases long. This is some 95% of the complete mRNA, which is ≈2.2 kilobases long according to RNA blot analysis (Fig. 5). Therefore, only short noncoding regions must exist in this mRNA, of which some is poly(A). This is in contrast to the mRNA coding for rat liver peroxisomal
acyl-CoA oxidase, a protein with a similar mass of 72 kDa. The mRNA coding for this protein is \(~3.8\) kilobases long, and therefore only 52\% of its length codes for the protein (51).

Growth of C. tropicalis cells in alkanol medium leads to a proliferation of peroxisomes in the cells and to increased levels of certain peroxisomal proteins, especially those of the \(\beta\)-oxidation system (21, 24, 26, 52). Our results demonstrate that the increase in \(\beta\)-oxidation enzymatic activity is due at least in part to a striking increase in acyl-CoA oxidase mRNA. Whether other mechanisms such as translational control or increased stability of the protein are also working to increase the level of \(\beta\)-oxidation activity in alkanol-grown cells is unknown at present.

Knowledge of the molecular structure of acyl-CoA oxidase, as determined from an analysis of the clone encoding it, combined with the knowledge of the molecular structures of other peroxisomal polypeptides determined from other clones, should aid greatly in elucidating the mechanisms of which peroxisomal polypeptides target themselves specifically to the peroxisome.

While this manuscript was being prepared for publication, Kamiyano and Okazaki (53) reported experiments in which a strategy similar to our own was followed to isolate DNA encoding peroxisomal proteins in C. tropicalis, except that genomic DNA was cloned rather than cDNA. Comparison of the two types of DNA will be instructive.

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