Protein, Cytochrome c Peroxidase*}

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Bacterial clones containing the gene for yeast cytochrome $c$ peroxidase, a nuclearly encoded mitochondrial protein, were isolated from a recombinant DNA library using an in situ radioimmunological assay. Escherichia coli transformants with this cloned DNA, YEp13-CCP, occur in the recombinant pool at a frequency of $10^{-3}$ to $10^{-4}$. The YEp13-CCP plasmid contains approximately 3000 base pairs of yeast chromosomal DNA inserted into the Bam H1 site of the vector YEp13. The identification of this cloned DNA is based on 1) its ability to hybridize to, and to select, cytochrome $c$ peroxidase-specific mRNA, 2) its expression in E. coli cells, and 3) DNA sequence analysis of the yeast DNA insert. The selection scheme described here represents a simple, rapid method for the isolation of nuclear genes that encode mitochondrial proteins. This approach is particularly useful for cloning the genes of mitochondrial proteins for which well characterized mutants are not available.

A good deal of effort has been directed toward studying the yeast mitochondrial genome and its gene products (1). Emphasis has also been placed on characterizing the events involved with the biogenesis and energy-generating functions of mitochondria (2, 3). Although 90% of yeast mitochondrial proteins are coded for by nuclear DNA (4), little is known about the organization, regulation, or chromosomal location of the genes that encode these proteins. Several nuclear DNA mutations have been reported to alter the expression of mitochondrial proteins (5, 6). Only one of these, the OPI gene, has actually been demonstrated to contain structural gene information (7). The mechanism(s) by which nuclear gene products are incorporated into mitochondria has not been elucidated to any great extent.

The identification and the isolation of specific genes from cloned pools of yeast DNA based on their ability to complement well defined mutations in Escherichia coli and yeast proteins has become fairly routine in recent years (8-10). Genes encoding yeast proteins for which mutants have not been selected can be identified using in situ DNA, RNA, or antibody screening procedures that do not depend on functional gene expression (11).

Yeast cytochrome $c$ peroxidase is localized to the area between the outer and the inner mitochondrial membranes (12). This nuclearly encoded protein is synthesized both in vivo and in vitro as a larger molecular weight precursor that is processed to its mature size upon translocation into mitochondria (13). The isolation of defined yeast mutants with alterations in the nuclear gene that codes for this protein has not been reported. We have employed a simple in situ antibody screening procedure to identify the clones in a yeast recombinant DNA library that carry the gene for yeast cytochrome $c$ peroxidase. The radioimmunological assay utilized in this study (14) differs from those employed by other groups (15, 16) to monitor the expression of eukaryotic genes in E. coli in that this assay optimizes the detection of membrane-bound proteins.

The yeast DNA present in the clones isolated using this procedure was characterized with respect to size, restriction endonuclease cleavage sites, and partial DNA sequence. The identification of this DNA as the cytochrome $c$ peroxidase gene is based upon several independent lines of evidence. Antigenically recognizable cytochrome $c$ peroxidase peptides are present in E. coli cells that carry this particular piece of yeast DNA. Recombinant plasmid DNA that contains all or part of this cloned DNA can hybridize to, or select, cytochrome $c$ peroxidase specific mRNA, as determined by hybridization-selected-translation experiments. The nucleotide sequence of a portion of the cloned DNA was determined. This stretch of DNA codes for an 80-aminoacid long peptide whose sequence precisely matches that found in the mature cytochrome $c$ peroxidase (17).

The selection scheme used here is not dependent on the functional expression of a given gene product or on the existence of well defined yeast or E. coli mutants. It requires both that one have appropriately prepared antisera to the protein of interest and that some part of this protein be synthesized in E. coli. As such, this approach may have wider applicability to the study of nuclearly encoded mitochondrial proteins than those used previously.

**MATERIALS AND METHODS**

**Strains and Medium**

The following E. coli strains were used: C600, K12, and JM109 (18, 19). E. coli C600 was generously given to us by I. Hacia, Cold Spring Harbor Laboratories. The yeast strain D273-108 (ATCC 25657) was used in this study. Transformed E. coli cells were grown in the presence of 200 μg/ml ampicillin in either LB media (19), TT media (20), or yeast extract, 0.1% glucose) or 2% galactose, 0.5% glucose, or 2% glucose. Yeast strains were propagated at 30°C in either YPD (1% yeast extract, 1% glucose, 2% agar) or in rich medium as described by Naececchini et al (25). Plasmid DNA was isolated from bacterial cells by standard techniques (26). Plasmid DNA was prepared from D273-108 cells grown in rich medium as described by Naececchini et al (25). Plasmid DNA was isolated from bacterial cells by standard techniques (26).

The "Materials and Methods" are presented in miniprint as prepared by the authors. Miniprint is easily read with the aid of a standard magnifying glass. Full size copies are available by request. The Journal of Biological Chemistry, 9560 Rockville Pike, Bethesda, MD 20814. Request Document No. 28C-291, cite the authors, and include a check or money order for $2.00 per set of photocopies. Full size photocopies are also included in the microfilm edition of the Journal that is available from Waverly Press.
clone bank. It is likely that these recombinants were isolated by amplification of a single transformant in the original clone bank.

**RESULTS**

Approximately 15,000 clones from a YEpi3-yeast DNA clone bank were screened with an immunoselected cytochrome c peroxidase antisera using a modified version of the Henning et al. (14) antibody screening procedure. The original protocol was altered to reduce background problems and to enhance sensitivity (see "Materials and Methods"). Putative peroxidase-expressing transformants occur in the pool at a frequency of 2-3 \times 10^{-3}. These clones were purified away from contaminating clones by selection with three sucrose-based media.

In vivo experiments outlined in this study were performed in accordance with the Guidelines of the National Institutes of Health for recombinant DNA research.

A schematic diagram of the restriction map determined for YEpi3-CCP 19 insert DNA is presented in Fig. 1. The 3.0-kb insert cannot be cleaved out of the vector DNA using BamHI because the ligation of this Sau3A restriction fragment into the BamHI site of YEpi3 failed to regenerate functional BamHI cleavage sites. This phenomenon has been observed by others (32).

The 1.7-kb HindIII digestion product of YEpi3-CCP 19 DNA digested in Fig. 1 was ligated into the HindIII cloning site of plasmid pBR322 and transformed into competent E. coli Drosina cells. This HindIII fragment contains 346 base pairs of pBR322 DNA (---) and 1.3 kb of yeast DNA (■). The results of additional restriction endonuclease analyses performed on this fragment are also indicated.

The expression of YEpi3-CCP 19 insert DNA in *E. coli* was examined by probing *in vivo* labeled bacterial lysates with antibodies to cytochrome c peroxidase. The plasmid-encoded proteins in *E. coli* cells (C600 or R11) transformed with either vector (pBR322, YEpi3) or recombinant (pCCP 19A, YEpi3-CCP 19) plasmid DNAs were identified using the maxicell labeling technique (31, 32). The cytochrome c peroxidase antibodies in extracts prepared from cells labeled in this manner were immunoprecipitated with total cytochrome c peroxidase antiserum, as indicated under "Materials and Methods." The results obtained upon electrophoresis of these cytochrome c peroxidase-specific proteins are presented in Fig. 2.

Maccacini et al. (13) have previously shown that the molecular weight of *in vitro* synthesized cytochrome c peroxidase (approximately 39,000) is equivalent to that of the *in vivo* synthesized precursor form of the protein. This precursor is about 6,000 daltons larger in size than the mature protein. Gasser et al. (33) have observed a third form of the protein which is intermediate in size to these proteins. These authors postulate a two-step mechanism for the conversion of the precursor cytochrome c peroxidase protein to its mature form. Two plasmid-dependent proteins in a maxicell lysate from YEpi3-CCP 19 transformed cells are recognized by cytochrome c peroxidase antiserum. These bands are indicated as 1.3 kb of yeast DNA (■).
Isolation of the Yeast Cytochrome c Peroxidase Gene

Fig. 2. Expression of YEp13-CCP 19 insert DNA in E. coli cells labeled in vivo with $^{35}$S-methionine. Plasmid DNA-dependent proteins were labeled in vitro using the maxicell technique (see "Materials and Methods"). Proteins recognized by cytochrome c peroxidase specific antiserum were immunoprecipitated from lysates of $^{35}$S-methionine labeled E. coli cells containing the following plasmid DNA: YEp13-CCP 19 (a, b, and g), no plasmid (c), pBlR222 (d), pCCP 19A (e), or YEp13 (f). The proteins in a and b were precipitated from lysates of cells labeled for 2-h and 30-min periods, respectively. In all other cases, cells were labeled for 1 h. Cytochrome c peroxidase precursor protein (h) was prepared by immunoprecipitation from in vitro synthesized total yeast RNA translation products.

Fig. 3. In vitro translation of hybridization-selected mRNA. Total yeast RNA (5 absorbance units) was incubated with 5–10 μg of restricted plasma DNA bound to nitrocellulose filters, as described under "Materials and Methods." The RNA that hybridized to the filter-bound DNA was eluted and translated in vitro in 20 μl of a nuclease-treated rabbit reticulocyte lysate reaction mixture containing $^{35}$S-methionine. One half of the translation products obtained were prepared directly for electrophoresis. The other half was immunoprecipitated with total antiserum prepared against mature cytochrome c peroxidase. The immunoprecipitates (a–e) and the total translation products (f–j) were electrophoresed on the same 10–15% polyacrylamide gel in the presence of 0.1% sodium dodecyl sulfate. An autoradiograph of the fluorographed gel is shown. The proteins obtained upon translation of mRNA selected by pCCP 19A DNA (a and i), YEp13 DNA (b and h), and YEp13-CCP 19 DNA (c and g) may be directly compared to the translation products resulting from residual endogenous mRNA in 10 μl of the reticulocyte lysate used in this experiment (f). The control lanes (d and e) correspond to cytochrome c peroxidase immunoprecipitates from translation of total yeast RNA and no exogenously added RNA, respectively.

The hybridization-selection-translation technique of Ricciardi et al. (27) was used to localize the yeast cytochrome c peroxidase gene to YEp13-CCP 19 insert DNA. Nitrocellulose-bound YEp13-CCP 19, and pCCP 19A DNAs were hybridized to total yeast RNA. The RNA that bound to the DNA immobilized on these filters was eluted and translated in vitro in a nuclease-treated rabbit reticulocyte lysate system (28). The total translation products and the cytochrome c peroxidase immunoprecipitable proteins from these translations are shown in Fig. 3, f–j and Fig. 3, a–e, respectively. A major protein corresponding in size to the precursor form of cytochrome c peroxidase (Fig. 3d) is obtained upon translation of RNA selected by hybridization either to YEp13-CCP (Fig. 3, g and f) or to pCCP 19A (Fig. 3i). The lower intensity of the peroxidase band in Fig. 3f as compared to that in Fig. 3g is explained by the use of one half the amount of DNA used to select mRNA in the former case. The protein that co-migrates with the cytochrome c peroxidase precursor is immunoprecipitable with antiserum to the mature peroxidase protein (Fig. 3, a and c). A protein that co-migrates with the cytochrome c peroxidase precursor was not detected among the total translation products of RNA selected by YEp13 DNA (Fig. 3h) or among the residual endogenous reticulocyte lysate products (Fig. 3f). Cytochrome c peroxidase immunoprecipitable proteins were not present in these translation products (compare Fig. 3, b and e to h and f, respectively).
for the 29 COOH-terminal amino acids of the mature cytochrome c peroxidase gene. This nucleotide sequence translates exclusively to mRNA, suggests that YEp13-CCP 19 insert DNA also codes for the 80 amino acids that are identical to the published amino acid sequence of positions 184-264 in the mature yeast cytochrome c peroxidase protein (17). These nucleotides can be obscured by an acid-hydrolyzable endogenous reticulocyte lysate band that migrates at about the same position in this gel.

These experiments show that the 3.0-kb yeast DNA insert in YEp13-CCP 19 contains all or part of the cytochrome c peroxidase gene. Some of this gene lies within the 1.3-kb portion of yeast DNA in the sub-clone pCCP 19A, since this DNA is able to specifically hybridize (hybridize to) cytochrome c peroxidase mRNA from a total population of yeast RNAs. Using the same criteria, we have found that the other 1.7 kb of the original insert also contains a portion of the peroxidase gene (data not shown).

Nucleotide sequencing data confirms that both YEp13-CCP 19 and pCCP 19A contain a segment of the yeast cytochrome c peroxidase gene. The same HindIII restriction endonuclease fragment that was cloned into pCCP 19A was also ligated into M13mp9 replicative form DNA in preparation for DNA sequence determination by the dideoxy chain termination method (22, 23, 34). This DNA sequence, and the amino acids it corresponds to in the mature cytochrome c peroxidase protein (17), is shown in a, b, the location of this nucleotide sequence within the YEp13-CCP 19 yeast DNA insert (c) is indicated by the arrow. The proposed position of the entire cytochrome c peroxidase gene is also shown (d).

Unknown. These proteins do not react with cytochrome c peroxidase antiserum (Fig. 3a). The presence of the 45,000-dalton yeast leu 2 gene product (7) among the YEp13 and YEp13-CCP 19-selected RNA translation products is probably obscured by an acid-hydrolyzable endogenous reticulocyte lysate band that migrates at about the same position in this gel.

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The cytochrome c peroxidase coding sequence in YEp13-CCP clones is expressed in E. coli as determined both by the Henning et al. (14) in situ radioimmunological assay, and by immunoprecipitation of proteins in YEp13-CCP 19 E. coli transformant cell lysates with antisera to mature cytochrome c peroxidase. Two peptides with cytochrome c peroxidase antigenic determinants were detected in these transformed cell lysates. The sizes of these proteins are similar to the intermediated processed and the mature form of cytochrome c peroxidase found in vivo in yeast cells (13, 33). We do not yet know if the multiple cytochrome c peroxidase peptides in YEp13-CCP 19 transformants were generated by specific proteolytic cleavage or degradation events, premature transcriptional or translational termination, or transcription from multiple bacterial or yeast promoters. We are currently conducting experiments to distinguish between these possibilities.

A combination of hybridization, nucleotide sequencing, and restriction endonuclease analyses were used to confirm the identity of the cloned gene and to localize this gene within the 3.0-kb yeast DNA insert of YEp13-CCP 19 plasmid DNA. The origin of the promoter used for transcription of this gene in E. coli has not been ascertained. The distance of the peroxidase gene from a promoter in the vector, however, rules out that transcription initiates at a position within the insert DNA. Our data indicate that the cloned DNA contains a large portion of the yeast cytochrome c peroxidase gene, but we do not yet know if the gene is present in its entirety. Nonetheless, the YEp13-CCP 19 clone will be useful for probing the transcriptional and translational regulation of cytochrome c peroxidase. We will also be able to use this plasmid DNA to prepare relatively large quantities of cytochrome c peroxidase-specific mRNA, and consequently, of the precursor form of the protein, in order to study the translation of this protein into mitochondria.

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REFERENCES
Isolation of the Yeast Cytochrome c Peroxidase Gene

Isolation of the yeast nuclear gene encoding the mitochondrial protein, cytochrome c peroxidase.
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