Isolation of the Yeast Nuclear Gene Encoding the Mitochondrial 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-CCPF, occur in the recombinant pool at a frequency of $10^{-5}$ to $10^{-4}$. The YEp13-CCPF plasmid contains approximately 3000 base pairs of yeast chromosomal DNA inserted into the BamH1 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 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

Strain and Media

The following E. coli strains were used: 5909, H11, and JM101 (18, 19). E. coli C600 was generously given to us by J. Hicks, Cold Spring Harbor Laboratories. The yeast strain D273-10B (20) was used in this study. D273-10B cells were grown in the presence of 300 μg/mL ampicillin in rich (2%) yeast extract, 1% Bacto-peptone, and 0.5% NaCl. Plasmid DNA was isolated from yeast cells using a glass bead disruption technique (21).

Yeast DNA preparation

Total yeast RNA was prepared from D273-10B cells grown to mid-logarithmic phase in rich medium as described by Nucenecchi et al (25). Plasmid DNA was isolated from bacterial cells prepared by the authors. Miniprint is easily read with the aid of a standard magnifying glass. Full size copies are available from the Journal of Biological Chemistry, 9650 Rockville Pike, Bethesda, MD 20814. Request Document No. 82C-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 this Journal that is available from Waverly Press.
found in the vector, YEp13. It is likely that these recombinants and to enhance sensitivity (see "Materials and Methods").

**Putative peroxidase-expressing transformants occur in the original protocol was altered to reduce background problems** (29). The clones carry a 3.0-kb DNA insert which is not shown). The four clones contain the same DNA on the 5-20 ESRI were ligated into the Bam HI closing site of the vector. Transformants of E. coli 5000 that could select these hybrid plaques are resistant to ampicillin, resistant to IPTG and X-gal (23). Plaques formed by phages containing the Hind III DNA restriction fragment were identified by color.

The expression of YEp13-CCP 19 insert DNA in E. coli was examined by probing in vitro labeled bacterial lysates with antibodies to cytochrome c peroxidase. The plasmid-encoded proteins in E. coli cells (C600 or RR1) transformed with either vector (pBR322, YEp13) or recombinant (pCCP 19A, YEp13-CCP 19) plasmid DNAs were identified using the maxicell labeling technique (31, 32). The cytochrome c peroxidase-specific proteins are presented in Fig. 2.

**RESULTS**

Approximately 15,000 clones from a YEp13-yeast DNA clone bank were screened with an immunoselected cytochrome c peroxidase antiserum 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 X 10^4. These clones were purified away from contaminating clones by selection with three successive antibody screenings.

**Four clones, designated YEp13-CCP 12, 15, 19, and 22, were chosen for further analysis and characterization of their yeast DNA insert**. All four clones contain the same DNA on the basis of restriction endonuclease mapping criteria (data not shown). The clones carry a 3.0-kb DNA insert which is not found in the vector, YEp13. It is likely that these recombinants arose by amplification of a single transformant in the original clone bank.

**The abbreviations used are**: kb, kilobase; CCP, cytochrome c peroxidase; PBS, phosphate-buffered saline; IPTG, isopropyl-1-thiogalactopyranoside; Xgal, 5-bromo-4-chloro-3-indolyl-β-D-galactoside.

Two schematic diagrams of the restriction map determined for YEp13-CCP 19 yeast DNA insert (c) and the restriction sites within this insert are indicated in a. A 1.7-kb HindIII fragment isolated from YEp13-CCP 19 (b) was subcloned into pBR322 and M13mp9 plasmids. This HindIII fragment consists of 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.

**A schematic diagram of the restriction map determined for YEp13-CCP 19 yeast DNA insert DNA is presented in Fig. 1. The 3.0-kb insert cannot be cleaved out of the vector DNA using **Bam** H1 because the ligation of this Sau3A restriction product into the **Bam** H1 site of YEp13 failed to regenerate functional **Bam** H1 cleavage sites. This phenomenon has been observed by others (32).

**The 1.7-kb HindIII digestion product of YEp13-CCP 19 DNA diagrammed in Fig. 1 was ligated into the HindIII cloning site of pBR322 and M13mp9 plasmids. This HindIII fragment contains 346 base pairs of pBR322 DNA in addition to 1.3 kb of the YEp13-CCP 19 yeast DNA insert.**

**The expression of YEp13-CCP 19 insert DNA in E. coli was examined by probing in vitro labeled bacterial lysates with antibodies to cytochrome c peroxidase. The plasmid-encoded proteins in E. coli cells (C600 or RR1) transformed with either vector (pBR322, YEp13) or recombinant (pCCP 19A, YEp13-CCP 19) plasmid DNAs were identified using the maxicell labeling technique (31, 32). The cytochrome c peroxidase peptides in extracts prepared from cells labeled in this manner were immunoprecipitated with total cytochrome c peroxidase antisem, as indicated under "Materials and Methods." The results obtained upon electrophoresis of these cytochrome c peroxidase-specific proteins are presented in Fig. 2.
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Fig. 2. Expression of YepI3-CCP 19 insert DNA in E. coli cells labeled in vivo with $^{35}$S-methionine. Plasmid DNA-dependent proteins were labeled in vivo 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: YepI3-CCP 19 (a, b, and g), no plasmid (c), pBlu322 (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.

Cytochrome c peroxidase antibodies (Fig. 2, a and g). These proteins are present in almost equivalent amounts in the lysate. Both peptides are smaller than the in vitro synthesized precursor form of the protein (Fig. 2h) by 2,000 and 5,000-6,000 daltons, respectively. These immunoprecipitated proteins were not observed when the labeling period was reduced to 30 min (Fig. 2b). This probably reflects a technical limitation of the maxicell labeling procedure. Increasing the labeling period from 1 to 2 h (see Fig. 2a) did not result either in increased incorporation or in substantial proteolytic degradation. A major cytochrome c peroxidase immunoprecipitable protein that corresponds in size to the smallest of these peptides is also found when YepI3-CCP 19 transformants are labeled and lysed using an alternate procedure that does not involve UV irradiation or amino acid starvation (data not shown). In this case, the protein is observed both after a 2- and a 20-h labeling period. The relationship between the two peroxidase precipitable peptides in YepI3-CCP 19 cell lysates or between these proteins and those seen by Gasser et al. has not yet been determined. No cytochrome c peroxidase cross-reacting material is found in cell lysates of the host strain (Fig. 2c) or of strains containing vector DNA (Fig. 2, d and f). We have not been able to identify cytochrome c peroxidase immunoprecipitated peptides in lysates prepared from pCCP 19A transformed E. coli RRII cells by either the maxicell technique (Fig. 2e) or by the alternate method alluded to above (data not shown). This may be due to subtle host strain differences (E. coli C600 versus RRII), preferential degradation of the partial peroxidase gene product that would result from the cytochrome c peroxidase mRNA encoded by pCCP 19A, or a failure to transcribe or translate the subcloned yeast DNA as a result of some anomaly in the primary or secondary structure of the insert DNA in this particular plasmid.

The hybridization-selection-translation technique of Ricciardi et al. (27) was used to localize the yeast cytochrome c peroxidase gene to YepI3-CCP 19 insert DNA. Nitrocellulose-bound YepI3-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 YepI3-CCP (Fig. 3, g and j) 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 antisera 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 translation products of RNA selected by YepI3 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).

The identities of the other protein bands resulting from the translation of YepI3-CCP 19-selected mRNA (Fig. 3g) are...
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Gly Tyr Glu Gly Pro Tyr Glu Ala Ser Val Phe Thr Arg Met Tyr Leu Arg
Glu GAA CAA GCG TGT CCG UGA GCA GCG GCT AAC AAC TCT TTC TGC TAC
200

Leu Arg Ala Gln Asp Tyr Leu Leu Gln Asp Arg Ala Gly Gly Gln Tyr Ser
TTT GTT AGT CAG CCA GCG TGG GGG GAC GCG GAA GGG AAC GAC GCA TCG GCA
190

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 antiserum 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 intermediately 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, suggests 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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