Elucidation of the Deficiency in Two Yeast Coenzyme Q Mutants

CHARACTERIZATION OF THE STRUCTURAL GENE ENCODING HEXAPRENYL PYROPHOSPHATE SYNTHETASE*

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The assembly of a polyisoprenoid side chain and its transfer to para-hydroxybenzoate are the first two steps of coenzyme Q biosynthesis. In yeast these reactions are catalyzed by hexaprenyl pyrophosphate synthetase and PHB:polyisoprenyltransferase, respectively. We have screened nine complementation groups of yeast coenzyme Q mutants for the activities of these two enzymes and found two strains deficient in either activity. The strain deficient in hexaprenyl pyrophosphate synthetase activity, C296-LH3, is complemented by the plasmid pG3/T1. When C296-LH3 was transformed with a shuttle vector containing a 2,187-base pair fragment from the genomic insert of pG3/T1, both glycerol growth and hexaprenyl pyrophosphate synthetase activity were restored. The activity of the latter enzyme was higher than that seen in wild-type yeast. The increase in activity could be attributed to a gene dosage effect of the multi-copy plasmid. A 1,419-base pair open reading frame encoding a 52,560-dalton protein was found on the genomic fragment. The size of the RNA transcript and the location of transcriptional initiation indicate that the entire open reading frame is contained within the mRNA.

Comparison of the hexaprenyl pyrophosphate synthetase amino acid sequence with amino acid sequences from the related enzyme farnesyl pyrophosphate synthetase show the presence of three highly conserved domains. Within two of the domains is an aspartate-rich motif found invariantly in the amino acid sequences of farnesyl pyrophosphate synthetase from three species and the hexaprenyl pyrophosphate synthetase amino acid sequence reported here. These aspartic acid motifs may comprise binding sites for the allylic and homoolytic substrates. The hydrophobicity profiles of the hexaprenyl pyrophosphate synthetase sequence and the farnesyl pyrophosphate synthetase sequence from rat appear similar. Furthermore, the hydrophobicity correlation coefficient of the comparison of these two sequences indicate with a high degree of confidence (p < 0.001) that the two proteins will fold into similar three-dimensional structures.

The isoprenoid biosynthetic pathway is responsible for the synthesis of a variety of products including sterols, dolichols, and coenzyme Q. Interestingly, the branches of the pathway leading to these three products each contain polyisoprenyl synthetase enzymes which catalyze a trans-4 condensation reaction between 5 carbon isoprene units (for review see Poulter and Rilling, 1981). The most active and certainly the best understood of these enzymes is farnesyl pyrophosphate synthetase. This enzyme catalyzes the trans-addition of two molecules of isopentenyl pyrophosphate onto dimethylallyl pyrophosphate to form farnesyl pyrophosphate. The polyprenyl synthetase activity of dolichol biosynthesis catalyzes the cis addition of between 8 and 20 isopentenyl pyrophosphate molecules onto farnesyl pyrophosphate. In contrast, the polyisoprenyl synthetase activity of coenzyme Q biosynthesis catalyzes the formation of all trans-polyisoprenyl pyrophosphates generally ranging in length of between 6 and 10 isoprene units depending on the species (Olson and Rudney, 1983).

Coenzyme Q functions as a lipid-soluble electron transporter between lipoprotein complexes of the mitochondrial respiratory chain. As originally proposed (Green, 1966), coenzyme Q is thought to shuttle electrons from NADH dehydrogenase (Complex I) and succinate dehydrogenase (Complex II) to reduce the bc1 complex (Complex III). The rate of electron transfer between the dehydrogenases and the bc1 complex was shown to be limited by coenzyme Q diffusion which is in turn determined by the length of the isoprenoid side-chain (Schneider et al., 1982). Perturbations in the mitochondrial membranes by detergent (Nishino and Rudney, 1977) or by viral infection (Casey and Bliznakov, 1973) were shown to alter the length of the isoprenoid side-chain of the coenzyme Q molecule. As with quinone molecules from other electron transport systems, coenzyme Q homologues are classified on the basis of the number of isoprene units in their polyisoprenoid moieties. The particular homologue present in an individual is thought to be a species-specific phenomenon but tissue-specific differences have been noted (Nazir and Magar, 1964). Although heterologous coenzyme Q homologues can function in vitro, changes in the specificity of the polyisoprenyl synthetase could in theory alter electrochemical processes.

Although normally acquired through dietary intake, the major source of coenzyme Q is derived from de novo synthesis and at least one human disease has been attributed to a defect in its biosynthesis (Ogasahara et al., 1989). In contrast to higher eukaryotes, yeast can grow aerobically by oxidative phosphorylation or anaerobically by the fermentation of glucose. This has enabled investigators to isolate mutant strains which are deficient in specific metabolic steps. Tzagoloff et al. (1975, 1990) isolated nine separate complementation groups of yeast which were defective in coenzyme Q. Presumably, these strains are deficient in specific steps of coenzyme

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13157
Q biosynthesis. In this paper we report the identification of the deficiency in two of the nine complementation groups of coenzyme Q mutants. In addition, we describe the isolation and characterization of the structural gene encoding hexaprenyl pyrophosphate synthetase, the first committed enzyme of coenzyme Q biosynthesis.

**EXPERIMENTAL PROCEDURES**

**Materials**—The wild-type yeast strain D273-10B/A1 (α mel6) and the set of nine coenzyme Q mutants strains (Tzagoloff et al., 1975, 1990) as well as the plasmids pG3/T1 and YEp551 were graciously provided by Dr. A. Tzagoloff (Columbia University). The prenyltransferase assay substrates isopentenyl pyrophosphate and geranyl pyrophosphate were a gift from Dr. H. C. Rilling, (University of Utah). [1-^14C]Isopentenyl pyrophosphate (56 Ci/mmol) and [α-32P]ATP (>1000 Ci/mmol) were purchased from Amersham Corp. [5,25P]ATP (7000 Ci/mmol) was purchased from ICN Radiochemicals. p-Hydroxybenzoate (PHB) was purchased from Kodak. Thin layer chromatography was performed on Silica Adsorbosil H plates (No. 16385) with the set of nine coenzyme Q mutants strains (Tzagoloff et al., 1975, 1990) as well as the plasmids pGS/Tl and YEp351 were graciously provided from Bethesda Research Laboratories.

**Synthesis of [U-^14C]p-Hydroxybenzoate—**PHB was synthesized by the alkaline fusiion of [U-^14C]tyrosine by the method of Parson and Rudney (1964) with several modifications. 50 μCi of [U-^14C]tyrosine (450 Ci/mmol) was dissolved with unlabelled tyrosine to give an activity of 50 Ci/mol and blown to dryness under nitrogen. 34.8 μl of 10 N NaOH and 48.8 μl of 10 N KOH were added and the resulting solution was blown to near dryness under nitrogen. The fusion reaction was carried out for 4 min at 270 °C in a sand bath. The tube was allowed to cool to room temperature and the sample was then acidified by adding 104 μl of 25% H2SO4 on ice. The resulting gelatinous precipitate was extracted 3 times with 1.0 ml of ethyl acetate and the organic phases were combined and back extracted twice with 2.0 ml of water. The organic phases were combined and blown to dryness under nitrogen and the PHB was resuspended in 0.5 ml of ethanol. Recovery of [U-^14C]p-hydroxybenzoate from this procedure was greater than 98%. The purity of the synthetic product was checked by thin layer chromatography on Silica Gel H plates which were developed with isopropanol:ammonium hydroxide:H2O (10:2:70). All detectable radioactivity was contained in a single peak (Rf 0.51) which co-migrated with an authentic PHB standard.

**Preparation of Intact Mitochondria—**Mitochondrial preparations were isolated by the method of Lanz et al. (1977). Yeast were grown to mid to late log phase in YPD media containing 1% yeast extract, 2% bacto-peptone, and 2% D-glucose at 30 °C. All subsequent steps were carried out at 4 °C. The cells were first pelleted by centrifugation at 2,500 × g for 1 min and resuspended in buffer A (0.4 M sorbitol, 0.5 M mannitol, and 20 mM Tris-HCl, pH 7.4). The cells were centrifuged again at 2,500 × g for 1 min and resuspended in an equal volume of buffer B (buffer A diluted 2:1 (v/v) with water). Sterile glass beads were added to the cells at a ratio of 4:1 (v/v) to the packed cell volume. The cells were disrupted by vigorously shaking the tubes 2 min at room temperature. The glass beads and particulate matter were removed by centrifugation at 100 × g for 1 min and the supernatant fraction saved. The pellet was washed with buffer B and the supernatant fractions pooled. The supernatant was centrifuged for 1 min at 1,200 × g, the meniscus removed, and the supernatant fraction collected. The meniscus was centrifuged for 10 min at 10,000 × g and washed twice with H2O. Cells were resedimented at 2,500 × g for 1 min and resuspended in buffer A (0.4 M sorbitol, 0.6 M mannitol, and 20 mM Tris-HCl, pH 7.4). The cells were centrifuged again at 2,500 × g for 1 min and resuspended in an equal volume of buffer B (buffer A diluted 2:1 (v/v) with water). The cell pellet was washed with buffer B and the supernatant fractions pooled. The supernatant was centrifuged for 1 min at 1,200 × g, the meniscus removed, and the supernatant fraction collected. The meniscus was centrifuged for 10 min at 10,000 × g and washed twice with H2O. Cells were resedimented at 2,500 × g for 1 min and washed once with 1 M sorbitol and resuspended in 10 ml of YEPP (1.0 mM sorbitol and 10 mM CaCl2), and finally resuspended in CaS at 50 °C and 150 mM CaCl2, and 10 mM Tris-HCl, pH 7.4) was added to the cells and let stand 5 min. Approximately 8 ml of regeneration top agar (30% Bacto-agar, 0.67% yeast extract, 0.05% Bacto-peptone, 1.0% sorbitol, 2% glucose, and the appropriate amino acid supplements) were added to the cells and then poured onto prewarmed selective plates. Leu+ transformants were isolated and tested for complementation by plating onto YPG (1% yeast extract, 2% bacto-peptone, and 2% glucose).

**Subcloning and DNA Sequence Analysis**—The plasmid pG3/T1 was isolated from a plasmid genomic library of wild-type nuclear DNA by virtue of its ability to restore growth on glycerol-containing media and thus, complement C296-LH3. The library was created by ligating a partial Sau3AI digest of wild-type nuclear DNA into the BamHI site of the shuttle vector YEp15 (Brosch et al., 1979). To facilitate subcloning, an approximately 4.5-kb BamHI-Sal1 fragment containing the entire nuclear DNA insert was excised from pG3/T1 and subcloned into the BamHI-Sal1 sites of the shuttle vector YEp551 (Hill et al., 1986) to generate YEpG3 by standard methods (Maniatis et al., 1982). To determine if the encoded polypeptide was necessary for complementation, restriction enzymes whose sites were present once in the polylinker region and once in the insert were used to digest YEpG3. The resulting plasmids were then re-ligated and tested for their ability to complement. DNA sequence was determined by the dideoxynucleotide chain termination method (Sanger et al., 1977) using a T7 sequencing kit (Pharmacia LKB Biotechnology Inc.). The smallest clone which retained the ability to complement was sequenced in both directions using synthetic oligonucleotides primers (Research Genetics).

**Hexaprenyl Pyrophosphate Synthetase**

**Molecular Nucleotide Mapping**—The probe was synthesized by the polynucleotide kinase reaction (Sagi et al., 1986) of the 460-bp region between the sequencing primers C2 and U1 (see Fig. 4). Yeast genomic DNA served as a template for the reaction and generated a fragment which was then "F"-end-labeled with T4 polynucleotide kinase and digested with Avel to yield two fragments, 149 and 340 bp in length. The 340-

\* A. Tzagoloff, personal communication.
bp fragment, labeled on the strand complementary to the 5’ region of the coding strand of the open reading frame, was purified on a 5% polyacrylamide gel. Approximately 7 ng (2.8 x 10⁵ cpm) was hybridized to 20 μg of wild type yeast poly(A) RNA in buffer containing 80% formamide, 0.4 M NaCl, 40 mM PIPES, pH 6.4, and 1 mM EDTA (Berk and Sharp, 1977) at 45 or 52 °C for 4 h. A control was incubated at 52 °C without poly (A) RNA. The samples were then treated with G1 nuclease (200 units/ml) in buffer containing 50 mM sodium acetate, pH 4.5, 150 mM NaCl, 6 mM ZnCl₂, and 5 μg/ml denatured salmon sperm DNA for 40 min at 4 °C. Finally, the DNA was ethanol-precipitated and analyzed on a 6% acrylamide, 8 M urea sequencing gel.

**Primer Extension**—The synthetic oligonucleotide sequencing primer U1 is complementary to the coding strand of the open reading frame of the HPS gene and was [3P] end-labeled with T4 polynucleotide kinase to a specific activity of 9 x 10⁶ cpm/μg. Approximately 2 ng of the probe (1.6 x 10⁵ cpm) was hybridized to 9 μg of yeast wild-type poly(A) RNA and extended by avian myeloblastosis virus reverse transcriptase as described by Teruya et al. (1990). Products of the primer extension were analyzed on a 6% acrylamide, 8 M urea sequencing gel as above.

**Computer Analyses**—Comparative sequence analyses were performed with programs from the University of Wisconsin Genetics Computer Group (Devereux et al., 1984). Hydrophobicity plots were generated with the program PEPLOUT which displays hydrophobicity profiles by the method of Kyte and Doolittle (1982). Hydrophobicity correlation coefficients were calculated by the method of Sweet and Eisenberg (1989). For these analyses each amino acid is given a hydrophobicity value and individual pairs of amino acids from two aligned sequences are compared by Equation 1:

$$r_{ij} = \frac{\sum H_i H_j}{(\sum H_i)^2}$$

where H₁ and H₂ are the hydrophobicities of the i-th residues in sequences 1 and 2, respectively. A program was written in C to perform the calculations in Equation 1. To facilitate statistical analyses the yeast HPS amino acid sequence was randomized, while maintaining composition, and aligned under the same conditions with the rat FPS sequences. The ratio of the observed rᵢ value to the standard deviation of the mean rᵢ after 10 such randomizations and alignments was then used in a t test for significance.

**RESULTS**

Tzagoloff and co-workers (1975, 1990) isolated nine individual complementation groups of yeast that would not grow on glycerol unless exogenous coenzyme Q₂ was added to the media (Table I). Presumably, these strains are defective in the path of coenzyme Q biosynthesis. Our attempts to directly measure the activity of the polyprenyl synthetase enzyme of the pathway by looking for the synthesis of polyprenyl pyrophosphates were unsuccessful. Therefore, the activities of hexaprenyl pyrophosphate synthetase and PHB:polyprenyltransferase were determined by the formation of 3-polyprenyl-4-hydroxybenzoate (Casey and Threlfall, 1979). This method of analyzing both enzyme activities is facilitated by the apparent lack of specificity of PHB:polyprenyltransferase (Daves et al., 1967; Nishino and Rudney, 1977). Hence, in the absence of endogenous polyprenyl synthesis, PHB:polyprenyltransferase will alkylate PHB with any available allylic prenyl pyrophosphate.

**In Vitro Synthesis of 3-Polyprenyl-4-hydroxybenzoates by Sonicated Mitochondria**—Casey and Threlfall (1978) described the formation of 3-diprenyl-4-hydroxybenzoate and 3-hexaprenyl-4-hydroxybenzoate in vitro under a variety of conditions. The authors identified the products by differential isotope labeling as well as exhaustive chemical derivatizations. To confirm the formation of the same products in our assay system we carried out the experiment shown in Fig. 1 with wild-type yeast (D273-10B/A1) mitochondria. When the Mg²⁺ concentration of the assay is 3.7 mM only 3-hexaprenyl-4-hydroxybenzoate was formed from [14C]PHB (Fig. 1A). At a higher Mg²⁺ concentration, 33.3 mM, 3-hexaprenyl-4-hydroxybenzoate and 3-diprenyl-4-hydroxybenzoate are formed at an increased level reflecting an increase in PHB:polyprenyltransferase activity and possibly also hexaprenyl synthetase activity (Fig. 1B). Addition of [14C]IPP, in place of [14C]PHB results in ^1⁴C incorporation into 3-hexaprenyl-4-hydroxybenzoate but not into 3-diprenyl-4-hydroxybenzoate (Fig. 1C). This result was expected since IPP is involved in elongation of geranyl pyrophosphate in the synthesis of 3-hexaprenyl-4-hydroxybenzoate. 3-Diprenyl-4-hydroxybenzoate is formed by the condensation of exogenous geranyl pyrophosphate onto PHB by the action of PHB:polyprenyltransferase. Deletion of IPP from the incubation prevents the elongation of geranyl pyrophosphate. To confirm the formation of the same products in vitro, wild-type yeast (D273-10B/A1) mitochondria were sonicated by three 1-s bursts. 1.0 mg of mitochondria were sonicated by three 1-s bursts. 1.0 mg of mitochondria were sonicated by three 1-s bursts. 1.0 mg of mitochondria were sonicated by three 1-s bursts.

**TABLE I**

| Yeast coenzyme Q mutants | Strain | Strain previously reported from this group¹ |
|--------------------------|--------|--------------------------------------------|
| G3                       | C296   | E2-247                                      |
| G9                       | C9     | E2-71                                       |
| G10                      | C33    | E2-107                                      |
| G17                      | C83    | E-1590                                      |
| G33                      | C39    | E2-249                                      |
| G63                      | C96    | E1-237                                      |
| G64                      | C97    | F4-140                                      |
| G75                      | C130   | N9-57                                       |
| G119                     | C296   |                                             |

¹ From Tzagoloff et al. (1975).

² From Tzagoloff and Dieckmann (1990).

**FIG. 1.** In vitro synthesis of 3-polyprenyl-4-hydroxybenzoate by sonicated mitochondria. Intact mitochondria were isolated from the wild-type yeast strain D273-10B/A1 as described under “Experimental Procedures.” Immediately before the assay the mitochondria were sonicated by three 1-s bursts. 1.0 mg of mitochondria were sonicated in the presence of 100 mM NaH₂PO₄, pH 7.4, 4 μM [U-¹⁴C]PHB (150 Ci/mole), 150 μM trilithium isopentenyl pyrophosphate, 120 μM geranyl pyrophosphate, and 3.7 mM MgCl₂ in a volume of 200 μl for 2 h at 30 °C with the following exceptions: no changes (A); 33 mM MgCl₂ instead of 3.7 mM MgCl₂ (B); ¹⁴C-labeled PHB is replaced with unlabeled PHB and IPP is replaced with ¹⁴C-labeled IPP (C); IPP is omitted from the incubation (D). Samples were extracted overnight with chloroform and analyzed by TLC as described under “Experimental Procedures.” Chromatograms were run from left to right; the origin and solvent front for each scan are indicated by arrows. The structures of the prenylated hydroxybenzoate products are shown at the positions of migration. x axis tick marks designate 16 mm.
pyrophosphate to form hexaprenyl pyrophosphate, therefore, only geranyl pyrophosphate is available for addition to $^{14}$C PHB to form 3-diprenyl-4-hydroxybenzoate (Fig. 1D).

Screening the Yeast Coenzyme Q Mutants for HPS and PHB:Polyprenyltransferase Activities—Seven of the nine coenzyme Q mutant complementation groups showed a normal ability to synthesize 3-hexaprenyl-4-hydroxybenzoate (data not shown). Two complementation groups, however, were deficient in either HPS or PHB:polyprenyltransferase activity. Wild-type mitochondria (Fig. 2A) synthesize both 3-diprenyl-4-hydroxybenzoate and 3-hexaprenyl-4-hydroxybenzoate under the standard assay conditions (33 mM Mg$^{2+}$). The strain C33 was unable to synthesize any substituted hydroxybenzoates (Fig. 2B). This reflects an absence of any detectable PHB:polyprenyltransferase activity. In contrast, C296 synthesizes only 3-diprenyl-4-hydroxybenzoate reflecting an absence of HPS activity (Fig. 2C). Since the HPS enzyme is nonfunctional, the strain C296 is unable to elongate geranyl pyrophosphate. Therefore, under these conditions the enzyme PHB:polyprenyltransferase transfers the only available allylic pyrophosphate (geranyl pyrophosphate) to PHB to form 3-diprenyl-4-hydroxybenzoate. This situation is analogous to the omission of IPP from the assay with wild-type mitochondria (Fig. 1D). Based on these results we have named the two loci which are required for these reactions coq1 and coq2 to reflect that they are implicated in the first and second steps of coenzyme Q biosynthesis, respectively. Thus, C296 which is deficient in HPS carries the coq1 allele and C33 which is deficient in PHB:polyprenyl transferase carries the coq2-1 allele.

Restoration of HPS Activity in C296-LH3—Transformation of C296-LH3 with the plasmid pG3/T1 restores growth on glycerol-containing media, implying that the strain has a competent electron transport chain and thus, functional coenzyme Q. To determine whether the ability of C296-LH3 harboring pG3/T1 to grow on glycerol was accompanied by wild-type HPS activity, assays to detect the formation of 3-hexaprenyl-4-hydroxybenzoate were performed. Under the standard assay conditions wild-type mitochondrial membrane-rich fractions synthesize both 3-diprenyl-4-hydroxybenzoate and 3-hexaprenyl-4-hydroxybenzoate and serve as standards (Fig. 3A and Figs. 1B and 2A). The strain C296-LH3 is deficient in HPS activity and is only capable of synthesizing 3-diprenyl-4-hydroxybenzoate in vitro (Fig. 3B). Alternatively, when C296-LH3 is complemented by pG3/T1 to restore growth on glycerol, HPS activity is also restored as indicated by the formation of 3-hexaprenyl-4-hydroxybenzoate (Fig. 3C). The amount of radioactivity associated with the 3-hexaprenyl-4-hydroxybenzoate peak was approximately 2-fold higher in mitochondrial fractions from C296-LH3 harboring the plasmid pG3/T1 compared with that from wild-type yeast (data not shown). This is probably due to a gene dosage effect since pG3/T1 is a multi-copy plasmid containing the 2$\mu$m circle origin of replication.

Sequence of the HPS Gene—To locate the gene within the 4.5-kb genomic fragment contained in the plasmid YEpG3, complementation analyses were performed. The smallest region of DNA capable of complementing C296-LH3 was found to be a 2,187-bp SstI-BamHI restriction fragment. The entire coding region as well as the 5' regions were sequenced bidirectionally the entire length. The bold arrows show the regions sequenced with the M13 universal and M13 reverse sequencing primers which hybridized within the multiple cloning site of the host plasmid, YEp351. The standard arrows indicate region sequenced using synthetic oligonucleotides 17-mers, except in the case of U1 which was a 40-mer. The names of the sequencing primers used to obtain regional sequence information are indicated (Fig. 4 and Figs. 1B and 2A). The strain C296-LH3 is deficient in HPS activity and is only capable of synthesizing 3-diprenyl-4-hydroxybenzoate in vitro (Fig. 3B). Alternatively, when C296-LH3 is complemented by pG3/T1 to restore growth on glycerol, HPS activity is also restored as indicated by the formation of 3-hexaprenyl-4-hydroxybenzoate (Fig. 3C). The amount of radioactivity associated with the 3-hexaprenyl-4-hydroxybenzoate peak was approximately 2-fold higher in mitochondrial fractions from C296-LH3 harboring the pG3/T1 plasmid compared with that from wild-type yeast (data not shown). This is probably due to a gene dosage effect since pG3/T1 is a multi-copy plasmid containing the 2$\mu$m circle origin of replication.

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analyses where total yeast RNA from C296-LH3 and wild-type yeast was hybridized to the original 4.5-kb genomic fragment revealed a single RNA band approximately 1.5 kb in size (data not shown). To map the site of transcriptional initiation Sh nuclease protection and primer extension analyses were performed (Fig. 6). Extension of the primer Ul (see Fig. 4) resulted in the synthesis of two products, 248 and 253 bases in length (Fig. 6). When the 340-base Sh probe was hybridized to yeast poly(A)+ RNA two distinct bands are protected which are the same size as those generated from the primer extension experiment (Fig. 6). These two methods corroborate each other since the probe used in the primer extension experiment was the same as was used to synthesize the Sh probe. Taken together these results indicate there are two major start-sites of transcription located 30 and 35 base pairs upstream of the ATG signal (Fig. 5).

Amino Acid Comparison of Yeast HPS and Farnesyl Pyrophosphate Synthetase—Alignments of amino acid sequences between yeast HPS and rat FPS were carried out by the method of Needleman and Wunsch (1970). The comparison revealed 22% of the amino acids were identical and 45% were conservative substitutions. Although the global sequence identity was not significant, three domains showing an impressive sequence identity were apparent. Fig. 7 shows an alignment of the three domains with the yeast HPS and FPS from rat (Clarke et al., 1987; Teruya et al., 1990), human (Shoares et al., 1989; Wilkin et al., 1990) and yeast (Anderson et al., 1989). The schematic diagram indicates the location of the domains within the polypeptides as well as the location of the cysteine residues. The HPS amino acid sequence contains 5 cysteine residues while each FPS sequence contains 6 cysteines. Only 3 of the cysteines are conserved in FPS between the three species and their locations are indicated in Fig. 7.

Structural Comparisons—Initial hydrophobicity comparisons between the yeast HPS and rat FPS showed similar profiles especially in the carboxyl-terminal 150 amino acids (Fig. 8). Since proteins which fold into similar three-dimensional structures have highly correlated hydrophobicities we sought to determine the degree of similarity of hydrophobicities between the yeast HPS and rat FPS amino acid sequences. The hydrophobicity correlation coefficient, rH, is calculated by comparing the hydrophobicities of each pair of amino acids from two aligned sequences (Sweet and Eisenberg, 1983). Table II shows the calculated rH values from alignments of the yeast HPS and the rat FPS sequence as well as the randomized yeast HPS sequence aligned with the rat FPS amino acid sequence. The rH value of 0.3 or greater from two properly aligned sequences indicates similar three-dimensional structures. Alignment of the HPS and FPS amino acid sequences produces an rH value of 0.3138 or 0.3055 depending on the hydrophobicity scale. This indicates that the two polypeptides will fold into similar structures. Align-
The sequencing reactions were carried out directly on the double-stranded plasmid pCM45 containing the 2187-base pair genomic fragment (lanes A, B, C, and G). For primer extension the 32P-labeled primer U1 was hybridized to poly(A') RNA and extended with avian myeloblastosis virus reverse transcriptase (lane PE). The S1 probe was synthesized by the polymerase chain reaction (see "Experimental Procedures") and hybridized in the presence (+) or absence (-) of poly(A') RNA at the indicated temperatures. b, base(s).

DISCUSSION

We have analyzed a set of yeast coenzyme Q mutants and found two strains defective in either the first or the second step of the coenzyme Q biosynthetic pathway. The strain C296 is deficient in hexaprenyl pyrophosphate synthetase activity and C33 is found to be deficient in PHB:polyprenyltransferase activity. These findings are based on the observation that neither strain can synthesize 3-hexaprenyl-4-hydroxybenzoate, a naturally occurring intermediate of coenzyme Q biosynthesis in yeast (Winrow and Rudney, 1969; Olson and Rudney, 1983). In our in vitro assay system the strain C296 synthesizes 3-diprenyl-4-hydroxybenzoate because it lacks functional FPS activity and is unable to elongate exogenous geranyl pyrophosphate by the classic 1'-4 condensation reaction. Therefore, geranyl pyrophosphate is the only aliphatic pyrophosphate available for PHB:polyprenyltransferase to add onto PHB. The strain C33 is unable to form any prenylated hydroxybenzoates despite the presence of all aliphatic pyrophosphates since it is deficient in PHB:polyprenyltransferase.

The inability to detect 3-triprenyl-4-hydroxybenzoate in our assay system is of interest since several groups (Momoese and Rudney, 1972; Poulter and Rilling, 1981) have detected FPS activity in mitochondria. This mitochondrial activity represents only a small fraction of the cytosolic FPS activity and may result from cytosolic contamination despite repeated washing of the mitochondria. In experiments from which we based our own assay system, Casey and Threlfall (1978) showed that dimethylallyl pyrophosphate, geranyl pyrophosphate, or farnesyl pyrophosphate served about equally well as aliphatic primers for the synthesis of 3-hexaprenyl-4-hydroxybenzoate. These authors showed that the formation of 3-triprenyl-4-hydroxybenzoate was detected only when farnesyl pyrophosphate was added exogenously. Furthermore, when mitochondria were incubated with farnesyl pyrophosphate at 33 mM MgCl2, the sole product observed was 3-triprenyl-4-hydroxybenzoate indicating that when farnesyl pyrophosphate is present (albeit at a saturating concentration) it will serve as a substrate for the PHB:polyprenyltransferase enzyme. Our experiments (Figs. 1–3) corroborate those of Casey and Threlfall (1978) in that the formation of triprenyl hydroxybenzoate was never observed when mitochondria were incubated with isopentenyl pyrophosphate (IPP), geranyl pyrophosphate, and PHB at high or low Mg2+ concentrations. There is a significant amount of FPS activity in our mitochondrial preparations relative to the rate of synthesis of polypropenyl hydroxybenzoates (data not shown). The inability to observe the synthesis of triprenyl hydroxybenzoate, especially in the FPS mutant C296, suggests that the FPS and PHB:polyprenyltransferase reactions may be coupled. This would enable these two enzymes to synthesize a single chain length polypropenyl hydroxybenzoate despite the presence of other available polypropenyl pyrophosphates and the inherent lack of specificity of PHB:polyprenyltransferase.

The structural gene encoding FPS was isolated from the plasmid pG3/T1 which was able to complement C296-LH4 and restore growth on glycerol. The gene was located on a 2187-base pair SstI-BamHI fragment of genomic DNA on the plasmid. When this region of DNA was transferred into C296-LH4 on a multi-copy plasmid the yeast acquired a glucorol® phenotype and regained FPS activity to a higher level than that observed in the wild-type strain D273-10B/A1. This probably resulted from a gene dosage effect and provided evidence that the SstI-BamHI fragment contained the structural gene for FPS.

The sequence of the 2187-base pair SstI-BamHI fragment revealed an open reading frame 1,419 base pairs in length coding for a 473-amino acid protein with a calculated molecular mass of 52,560 daltons. Approximately 150 base pairs upstream of the initiation ATG site is a poly(dA·dT) region which has been reported to be a common feature in the promoters of constitutively expressed yeast genes (Struhl, 1985). Also present in the sequence are two TATA-like sequences located 29 and 34 base pairs upstream of the transcriptional initiation site. In replacement and deletion studies on transcriptional initiation of the yeast HIs4 gene (Nagawa and Fink, 1985) and the CYCI gene (Hahn et al., 1985) it was reported that initiation takes place at particular initiation sequences which are located within a window of 60–120 nucleotides downstream of the TATA element. The two TATA-like sequences in the FPS 5' region appear to be located too close to the transcription start-site and whether they are functional in vivo remains to be determined. TATA elements are not an absolute requirement of yeast promoters. For example, the TRF3 promoter lacks the conserved TATAAAA sequence and instead contains a GCN4 binding site 28 nucleotides upstream of the mRNA start-site (Chen and Struhl, 1989). In the 3'-untranslated region of the FPS gene two polyadenylation signals are located 15 and 29 base pairs downstream from the termination codon TAA (Fig. 5).

Northern hybridization of yeast RNA probed with genomic...
The domains which display significant sequence conservation are shown diagrammatically and illustrate their relative positions within the yeast HPS and a consensus structure of FPS from rat, human, and yeast. The location of all cysteine residues (C) are shown in the yeast HPS while only conserved cysteines (present in the three species) are shown in the FPS consensus structure. Amino acid alignments are shown for the three domains. Conserved amino acid residues present in at least three out of four sequences are boxed.

**Fig. 8. Hydrophobicity profiles derived from the primary sequences of yeast HPS and rat FPS.** The amino acids are numbered from 1 to 354 (FPS) or 1 to 473 (HPS). Positive values represent hydrophobic amino acids and negative values indicate hydrophilic residues.

**TABLE II**

Comparison of hydrophobicity correlation coefficients between the yeast HPS and rat FPS sequences

| Alignment                      | Hydrophobicity scale | Consensus  
|-------------------------------|-----------------------|------------
| Yeast HPS × rat FPS           | 0.3138 (0.0443)       | 0.3055     
| Yeast HPS (randomized) × rat FPS| 0.2492 (0.0422)       | 0.2043 (0.0445) |

*Hydrophobicity scale from Dayhoff et al. (1979).*

*Hydrophobicity scale from Sweet and Eisenberg (1983).*

DNA capable of complementing C296-LH5, identified only a single mRNA species approximately 1.5 kb in length (data not shown). The RNA mapping studies confirmed that the 1419-base pair open reading frame of HPS (Fig. 5) is in fact transcribed and present in the mRNA population.

Polyprenyl synthetases have not received much attention and little is known about them in contrast to the cholesterogenic enzyme FPS. In all species examined, the polyprenyl synthetase involved in coenzyme Q synthesis is found to be a dimer and is located in the cytoplasm while HPS is membrane associated in mitochondria. The comparison did show, however, the presence of three highly conserved domains which were also present in the amino acid sequence of FPS from human and yeast (Fig. 7). A closer examination revealed the presence of two aspartate-rich sequences present in domains I and II of all four polyprenyl synthetase sequences (Fig. 7). The consensus sequence (I, L, or V)XDDXXD, where X can be any amino acid, was used to search the NBRF protein data base to look for similar sequences. Out of approximately 12,500 sequences we found the consensus sequence to occur in about 1% of the protein sequences or less than once in every 25,000 amino acids. We were not able to find any proteins which contained repeats of the consensus sequence other than the prenyl synthetases mentioned above. Based on the protein data base searches about one in every 10,000 proteins would be predicted to contain a repeat of the consensus sequence. The occurrence of a repeat of the aspartate-rich consensus sequence in all prenyl synthetase enzymes reported to date implies a functional and perhaps phylogenetic relationship exists between these proteins.

A common feature between HPS and FPS is the binding to both the homoallylic isoprenyl pyrophosphate and to an elongating allylic polyprenyl pyrophosphate. In binding experiments on FPS, King and Rilling (1977) reported evidence which strongly suggests that both the homoallylic and allylic substrates bind as their respective magnesium salts. We propose that the conserved aspartate repeats are involved in facilitating the binding of the substrates by forming magnesium salt bridges between the substrates and the catalytic site. There would be precedence for this type of ionic interaction. Brenner (1987) found a short conserved region containing an invariant pair of aspartic acid residues between the two ATP-binding enzymes aminoglycoside phosphotransferase and viomycin phosphotransferase. The author postulated that the aspartates were involved in forming magnesium salt bridges between the substrates and the catalytic site. These would be precursors for this type of ionic interaction.
quence stabilizing binding by interacting with the hydrocarbon tails of the allylic and homoallyllic substrates.

In addition to prenyl synthetase enzymes other enzymes which prenylate nonisoprenic substrates have been isolated. These prenyltransferase enzymes are involved in tRNA modification (Najarian et al., 1987) and cytokinin biosynthesis (Goldberg et al., 1984, Strabala et al., 1989). A search through these sequences for the presence of the aspartate consensus sequence was unsuccessful. This is not altogether surprising, though, since these enzymes must accommodate different electrophilic substrates which undoubtedly impose different steric requirements on the active sites. Of interest, however, was the finding that the yeast MOD5 amino acid sequence, an enzyme which prenylates tRNAs with dimethylallyl pyrophosphate (Najarian et al., 1987), contains 5 of the 7 invariant residues of Domain II, including the 3 aspartic acids (PEPLFQRMLDRVD). The common substrate of prenyl synthetases and non-isoprene prenylating enzymes is allylic pyrophosphate synthetase from Neurospora crassa and geranylgeranyl pyrophosphate synthetase.

The number of polyprenyl synthetase enzymes in vertebrates represents only a small fraction of that found in plants, fungi, and bacteria. In this respect, the identification of peptide domains critical to the 1'-4 condensation reaction may facilitate the isolation of other members of this diverse family.

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Note Added in Proof—We have recently learned from Drs. G. Macino and G. Morelli (Rome, Italia, manuscripts in preparation) that both farnesyli and geranylgeranyi pyrophosphate synthetase from Neurospora crassa share significant sequence identity with Domains I and II.

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