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Farnesyl Diphosphate Synthetase

MOLECULAR CLONING, SEQUENCE, AND EXPRESSION OF AN ESSENTIAL GENE FROM *SACCHAROMYCES CEREVISIAE**

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Farnesyl diphosphate (FPP) synthetase is a key enzyme in isoprenoid biosynthesis which supplies C_{15} precursors for several classes of essential metabolites including sterols, dolichols, and ubiquinones. The structural gene for FPP synthetase was isolated on a 4.5-kilobase *Eco*RI genomic restriction fragment from the yeast *Saccharomyces cerevisiae*. The clone encodes a 40,483-dalton polypeptide of 342 amino acids with a high degree of similarity to the protein encoded by a putative rat liver clone of FPP synthetase (Clarke, C. F., Tanaka, R. D., Svenson, K., Wamsley, M., Fogelman, A. M., and Edwards, P. A. (1987) *Mol. Cell Biol.* 7, 3138–3146) and to an active site protein fragment from avian liver FPP synthetase (Brems, D. N., Bruenger, E., and Rilling, H. C. (1981) *Biochemistry* 20, 3711–3718). When cloned into the yeast shuttle vector YRp17, the 4.5-kilobase *Eco*RI fragment directed a 2–3-fold over-expression of FPP synthetase activity in transformed yeast cells. The levels of expression were independent of culture growth phase and orientation of the insert, indicative of a functional promoter in the clone. Disruption of the FPP synthetase gene from a diploid yeast strain, followed by dissection and analysis of tetrads, demonstrates that the gene is an essential, single copy number gene in yeast. The gene for FPP synthetase resides on chromosome XI as judged from Southern blots of separated yeast chromosomes.

The major building steps in isoprenoid metabolism are catalyzed by prenyltransferases (Poulter and Rilling, 1981). These enzymes mediate alkylations of nucleophilic acceptors by potent allylic diphosphate electrophiles in reactions that are unique to the pathway. The 1′-4 condensation with isopentenyl diphosphate (IPP)[1] as an acceptor constitutes the trunk of the isoprenoid biosynthetic tree, and a family of 1′–4 prenyltransferases catalyze synthesis of polyisoprenoid diphosphates with discrete chain lengths and double bond stereochemistries (Poulter and Rilling, 1981). Other prenyltransferases catalyze reactions at branch points in the pathway, for example the c1′-2-3-cyclopropanations by squalene and phytoene synthetase that are the first pathway-specific transformations in sterologenesis and carotenogenesis, the alkylation of AMP by dimethylallyl diphosphate (DMAPP) in cytokinin biosynthesis, and the alkylation of tryptophan by dimethylallyl diphosphate in biosynthesis of ergot alkaloids.

Farnesyl diphosphate synthetase (EC 2.5.1.1) is the central enzyme in the 1′-4 chain elongation process (Poulter and Rilling, 1981) (Scheme 1). It catalyzes the sequential condensations of DMAPP and geranyl diphosphate with IPP. The product, farnesyl diphosphate (FPP), then partitions among several branches of the pathway. In eubacteria, common end products are respiratory quinones and bacterial dolichols (Fujisaki *et al.*, 1986). In eurkaryotes, respiratory quinones, dolichols, sterols, heme a, and modified polypeptides are among the further metabolites of FPP (Brown and Goldstein, 1980; Anderegg *et al.*, 1988).

FPP synthetase is also the best characterized of the prenyltransferases. The enzyme has been purified to homogeneity from several eukaryotic sources, including *Saccharomyces cerevisiae* (Eberhardt and Rilling, 1975), avian liver (Reed and Rilling, 1975), porcine liver (Barnard *et al.*, 1978; Yeh *et al.*, 1977), and human liver (Barnard and Popjak, 1981). In all of these organisms, the enzyme is a dimer of 80–84 kDa. The more extensively studied avian liver enzyme has two identical subunits, a motif that is apparently conserved in the enzyme from other eukaryotes. Each subunit has a single catalytic site that catalyzes condensation of IPP with both DMAPP and geranyl diphosphate. Mechanistic studies clearly point to an ordered addition of substrates, followed by an electrophilic condensation to join the isoprenoid units (Laskovics and Poulter, 1981; Poulter *et al.*, 1981).

As previously noted for IPP:DMAPP isomerase (Anderson *et al.*, 1989), there are no known mutants for FPP synthetase, and relatively little is known about regulation of the enzyme. Most reports involve studies of cholesterolgenesis in liver, where it has been demonstrated that activities for many of the enzymes catalyzing reactions between mevalonate and squalene, including FPP synthetase, exhibit 8–14-fold changes in activity during cholesterol fasting and feeding cycles (Slakey *et al.*, 1972). Recently Clarke and co-workers (Clark *et al.*, 1987), took advantage of these observations to isolate clones by differential binding of a rat liver cDNA library to radiolabeled cDNA probes prepared from rat liver mRNA isolated from animals having induced and repressed levels of cholesterol biosynthesis. They discovered an open reading frame which encoded a cholesterol-repressible 39,600-dalton polypeptide with a subunit molecular mass and an amino acid composition similar to those reported for FPP

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EMBL Data Bank with accession number(s) J05091.

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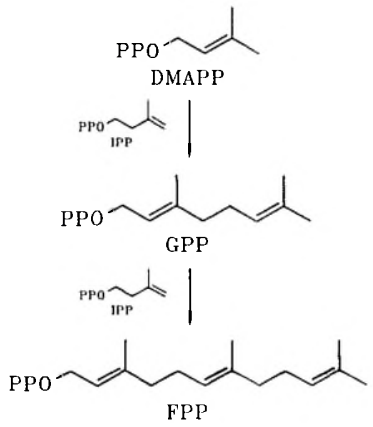
[1] The abbreviations used are: IPP, isopentenyl diphosphate; DMAPP, dimethylallyl diphosphate; FPP, farnesyl diphosphate; HPLC, high pressure liquid chromatograhy; kb, kilobase; CBI, codon bias index.



SCHEME 1

synthetase from chicken, pig, and human. In addition, a region of the amino acid sequence predicted by their clone showed a match of 17 out of 30 with an active site fragment from avian liver FPP synthetase obtained by photoaffinity labeling (Brems *et al.*, 1981). More recently, Ashby and Edwards (1989) reported that purified antisera directed against a fusion protein containing the amino-terminal region of bacterial anthranilate synthetase and the full length rat liver polypeptide inactivated rat liver cytosolic prenyltransferase activity.

We isolated the gene for FPP synthetase from yeast genomic DNA by hybridization with a synthetic probe based on the NH_2-terminal amino acid sequence of the enzyme from *S. cerevisiae*. A clone was obtained which encodes a 40,500-dalton protein that shows substantial sequence similarity with the Brems (Brems *et al.*, 1981) fragment from avian liver FPP synthetase and the polypeptide sequence reported by Clarke and co-workers (Clarke *et al.*, 1987). Transformants containing the gene in a yeast shuttle vector showed a 2–3-fold increase in activity for FPP synthetase. Experiments describing the cloning and expression of the yeast gene for FPP synthetase are discussed.

EXPERIMENTAL PROCEDURES

Materials—[1-^{14}C]Isopentenyl diphosphate was prepared from [1-^{14}C]isopentenyl alcohol (Du Pont-New England Nuclear) by the method of Davisson and Poulter (1986). Solvents were of reagent grade or better. Acrylamide, bisacrylamide, and urea for sequencing gels were of molecular biology grade. Radioactivity was measured in Optifluor scintillation media (Packard Instrument Co.) using a Packard Tricarb model 4530 liquid scintillation spectrometer.

Strains, Media, and Growth Conditions—Escherichia coli strains DH5 and JM101 were previously described (Anderson *et al.*, 1989). Yeast strains S288C (MATα, *mal, gal2*; D. Botstein) and JGY193 (*Gal*4C, *ura*3-52; this study), and JGY202 (MATa/MATα; *his3-A1/+; leu2-3, leu2-112/leu2; ura3-52/ura3; trp1-289/+; +/rad54-3; +hom3-10; +/his1; +/trp2*; this work) were used. Yeast strains were grown at 30 °C in YEPD media (Sherman *et al.*, 1986) for the purpose of obtaining genomic DNA or for growth under nonselective conditions. Those harboring plasmids were grown in SD synthetic complete minimal media (Sherman *et al.*, 1986) lacking uracil (*ura3* selection).

General Procedures—Restriction enzyme digestions, hydrolysis with *E. coli* alkaline phosphatase, transformations, and other standard molecular biology techniques were carried out as described by Maniatis *et al.* (1982). FPP synthetase assays were performed using the acid lability procedure (Johnson *et al.*, 1974; Reed and Rilling, 1975).

Purification of FPP Synthetase—All procedures were performed at 4 °C with the exception of the final Protein Pak® HPLC step. All buffers contained 10 mM 2-mercaptoethanol. FPP synthetase was purified from baker's yeast (*S. cerevisiae*, Westco Distributing Co., Salt Lake City, UT) by a modification of the procedure described by Eberhardt and Rilling (1975). Fresh baker's yeast (1.5 lb) was suspended in 1.5 liter of 100 mM sodium phosphate, 10 mM 2-mercaptoethanol, 1 mM phenylmethylsulfonyl fluoride, pH 7.0, and disrupted in a bead beater (Biospec Products, Bartlesville, OK). The material was processed in 125 ml portions in a 250-ml vessel using a 1:1 (v/v) ratio of cell suspension to beads. Cell breakage was achieved in 8 min using cycles of 15 s on and 15 s off to minimize heating. The beads were rinsed with two 100 ml portions of ice-cold buffer, which was combined with the extracts and adjusted to pH 5.0. The precipitated material was removed by centrifugation at 8000 × *g* for 40 min. Protein in this supernatant precipitating between 45 and 75% saturation with ammonium sulfate was collected and dialyzed against four changes of 10 liters of 5 mM sodium phosphate, 10 mM 2-mercaptoethanol, 1 mM phenylmethylsulfonyl fluoride, pH 7.0. The dialyzate was chromatographed on Whatman DE52 cellulose followed by chromatography on hydroxylapatite (Eberhardt and Rilling, 1975).

Active fractions from the hydroxylapatite column were combined and precipitated in 75% ammonium sulfate. The precipitate was collected by centrifugation, and the pellet was resuspended in 1 ml of 50 mM sodium phosphate, 10 mM 2-mercaptoethanol, pH 7.0, and dialyzed against four 4-liter changes of dialysis buffer. The resulting solution (9.0 ml) was further purified on a 0.9 × 27-cm chromatofocusing column (Pharmacia LKB Biotechnology Inc.), preequilibrated in 25 mM histidine, pH 6.3. The column was eluted at a flow rate of 0.33 ml/min with 200 ml of 1:8 diluted polybuffer, pH 4.5. A single sharp peak of activity eluted at an approximate pH of 5.0. The active fractions were pooled, precipitated in 75% ammonium sulfate, and collected by centrifugation. The pellet was resuspended in 50 mM sodium phosphate, 10 mM 2-mercaptoethanol, pH 7.0, and clarified by centrifugation for 10 min in a microcentrifuge. The solution was chromatographed on a 15 × 49-cm column of Superose 6B (Pharmacia LKB Biotechnology Inc.) which had been preequilibrated with 50 mM sodium phosphate, 150 mM KCl, 0.1 mM EDTA, pH 7.0, and was developed with the same buffer at a flow rate of 0.33 ml/min. Active fractions were pooled and concentrated against 25 mM sodium phosphate, 15 mM 2-mercaptoethanol, pH 7.0, using a Micro-ProDicon concentration-dialysis apparatus (Biomolecular Dynamics, Beaverton, OR).

Most remaining impurities were removed by chromatography on a 7.5 mm × 7.5-cm Protein Pak ion exchange HPLC column (Waters Associates). Separation was achieved by loading 0.55 mg of protein at 0.7 ml/min onto the column previously equilibrated in 9:1 (v/v) 5 mM sodium phosphate, pH 7.0 (buffer A): 100 mM sodium phosphate, pH 7.0 (buffer B). After a 5 min wash, the proportion of buffer B was increased linearly to 30% in 5 min, increased linearly to 60% buffer B in an additional 25 min, held at this point for 5 min, and increased linearly to 100% buffer B in an additional 10 min. FPP synthetase eluted at 40% B in approximately 40% yield. Active fractions were pooled and concentrated against 100 mM sodium phosphate, 2 mM dithiothreitol, 0.2 mM EDTA, pH 7.0, using a Micro-ProDicon concentration-dialysis apparatus. The protein isolated from this purification was judged to be greater than 90% pure by sodium dodecyl sulfate-gel electrophoresis.

Genomic Cloning of the Prenyltransferase Gene—A sample of the purified protein was submitted to the University of Illinois Biotechnology Center for NH_2-terminal Edman degradation. The first seven cycles of this analysis were ambiguous but, starting at position eight, yielded the partial amino acid sequence NH_2-Leu-Glu-Arg-Phe-Leu-Asn-Val-Phe-Pro-Lys-Leu-COOH. This sequence was used to construct a DNA probe based on yeast codon usage for a highly expressed gene (Bennetzen and Hall, 1982). The 33-mer 5′-d(TTG GAA AGA TTC TTG AAC GTT TTC CCA AAG TTG)-3′ was radioactively end-labeled with ^{32}P and used as a probe for the prenyltransferase structural gene.

Total yeast genomic DNA was isolated from *S. cerevisiae* strain S288C by the method of Sherman *et al.* (1986). Two μg were digested in each of 10 separate restriction digests using *Bam*HI, *Bst*EII, *Cla*I, *Eco*RI, *Hin*dIII, *Kpn*I, *Pst*I, *Sac*II, *Sal*I, and *Xho*I. The digests were run on a 1% agarose gel, blotted to nitrocellulose, and hybridized with 3 μg of the radioactive 33-mer as described by Yarger *et al.* (1986). Double digestions of genomic DNA were performed with the combinations *Eco*RI/*Sal*I, *Hin*dIII/*Sal*I, *Cla*I/*Sal*I, *Eco*RI/*Cla*I, *Eco*RI/*Hin*dIII, and *Hin*dIII/*Cla*I. The reaction mixtures were probed as described above in order to generate a crude genomic restriction map of the region where probe bound.

A 4.5-kb *Eco*RI fragment from the yeast genome which hybridized to our probe was cloned into pBR322 as follows. A digestion reaction containing 5 μg of S288C genomic DNA was run overnight on a preparative horizontal 1% agarose gel. The appropriate slices were placed in dialysis tubing and electroeluted. The DNA was recovered

by ethanol precipitation, washed with 70% ethanol, dried under vacuum, and resuspended in 20 μl of 10 mM Tris-HCl, 1 mM EDTA, pH 7.2. The solution was used directly in ligation reactions with pBR322 treated with *Eco*RI and bacterial alkaline phosphatase.

Approximately 3000 transformant colonies were screened by colony lift for the presence of plasmids bearing the genomic fragment hybridizing to the initial probe. Candidate colonies which hybridized to the radioactive probe were picked from the master plate and restreaked onto selective plates. Plasmid DNA was prepared by the boiling procedure of Maniatis *et al.* (1982). The plasmids were each digested with *Eco*RI and double digested with *Eco*RI/*Sal*I, *Eco*RI/*Hin*dIII, and *Eco*RI/*Cla*I. The digests were electrophoresed on 1% agarose, Southern hybridized, and probed as described above. A single colony was obtained which harbored plasmid pARC00, consisting of pBR322 bearing a 4.5-kb genomic insert complementary to the probe.

Expression of the Yeast FPP Synthetase Structural Gene—To demonstrate the presence of the full length prenyltransferase structural gene in clone pARC00, the 4.5-kb *Eco*RI genomic insert was ligated into a yeast compatible shuttle vector YRp17. Examples of each insert orientation were obtained (pARC01 and pARC02) and were used to transform yeast strain JGY193 to uracil prototrophy.

Yeast Transformations—Transformations of yeast were performed according to the modified lithium acetate procedure of Ito *et al.* (1983), as previously described (Yarger *et al.*, 1986). Transformants were restreaked on selective plates and verified as to their plasmid content by the procedure of Sherman *et al.* (1986). The transformed strains were grown to late log-phase in selective media and stored as 15% glycerol stocks at −60 °C.

Southern Hybridization of Yeast Chromosomal Blots—In order to locate the chromosome(s) encoding the FPP synthetase structural gene, a commercially available yeast chromosomal blot (Clontech Laboratories, Inc., Palo Alto, CA) was probed with the 4.5-kb clone, made radioactive by nick translation (Maniatis *et al.*, 1982).

Sequencing Methods—DNA sequence analysis of the prenyltransferase gene was performed with the dideoxy-chain termination method of Sanger *et al.* (1977) using Sequenase® and the associated DNA sequencing kit purchased from U. S. Biochemicals. Computer-assisted analysis of sequence results was performed using PC Gene software (Intelligenetics Inc., Mountainview, CA). Strain JM101 (Yanisch-Perron *et al.*, 1985) was used as described in the sequencing instruction manual from Bethesda Research Laboratories for transformations of M13 derived and phagemid (pBluescript SK(+)) based plasmids. Helper phage R408 (Stratagene) was used as described by the supplier to produce single-stranded phagemid DNA.

RESULTS

Purification of Farnesyl Diphosphate Synthetase from Yeast—Yeast FPP synthetase was purified by us using a modification of the procedure described by Eberhardt and Rilling (1975). In preliminary runs we noted that the enzymatic activity eluted from hydroxylapatite in two well-resolved fractions at approximately 90 and 110 mM phosphate. A similar phenomenon was reported for porcine liver FPP synthetase (Yeh and Rilling, 1977; Koyama *et al.*, 1977; Barnard *et al.*, 1978). Barnard and co-workers (Barnard *et al.*, 1978) discovered that the two forms interconverted upon treatment with 2-mercaptoethanol and oxidized glutathionine, and attributed differences in chromatographic behavior to intramolecular disulfide linkages. The yeast enzyme did not respond in a similar manner, although the ratio of the two peaks of activity varied upon addition of phenylmethylsulfonyl fluoride to the initial disruption buffer, suggesting that the enzyme was sensitive to proteolysis.

The first peak of activity eluted from hydroxylapatite just before the main protein band and was easier to purify. Unfortunately, Edman degradation of a 100-μg sample gave no signal, and we assume that the protein is blocked at its NH_2 terminus. Another preparation which contained the second peak gave a well-defined sequence but only after the eighth cycle. This sequence was used to design the DNA probe for the initial hybridization experiments.

Cloning of the Prenyltransferase Gene—The structural gene for FPP synthetase was cloned from yeast by construction of a partial genomic restriction map of the region surrounding the gene, followed by isolation of the appropriate restriction fragment. Using the amino acid sequence for FPP synthetase obtained as described above, we constructed a 33-base single stranded DNA probe based on preferred codon usage for a

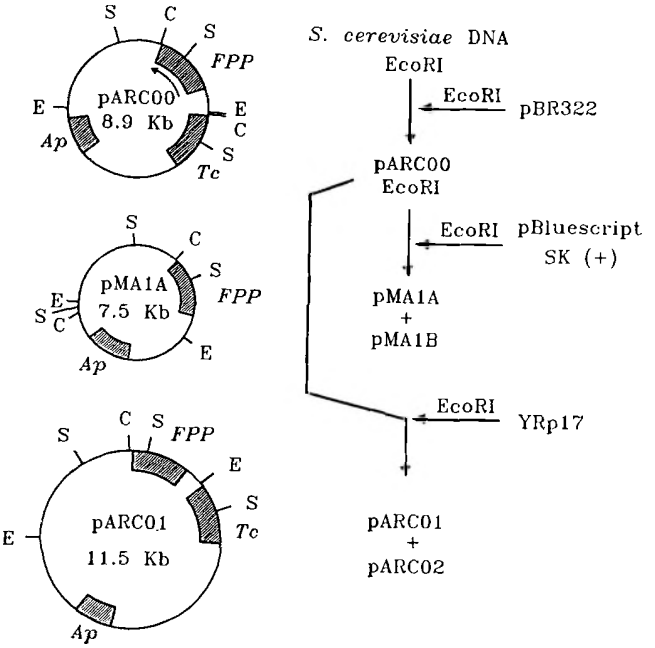


FIG. 1. **Construction of plasmids used in this study.** A 4.5-kb *Eco*RI fragment that hybridized to a synthetic DNA probe for the amino terminus of FPP synthetase was cloned from the genome of *S. cerevisiae* into pBR322 to yield plasmid pARC00. The *Eco*RI insert was then moved into the phagemid pBluescript SK(+) in order to generate template single-stranded DNA for sequencing. The two possible orientations of the insert were isolated, pMA1A and pMA1B, and only one is shown for clarity. The *Eco*RI insert of pARC00 was further cloned into the yeast shuttle vector pYRP17 as shown for expression of the gene in yeast. Again, two orientations of insert were obtained, pARC01 and pARC02, only one of which is shown. Plasmids are drawn to scale. *Arrows* adjacent to the FPP synthetase gene indicate direction of transcription. Abbreviations are as follows: *C*, *Cla*I; *E*, *Eco*RI; *S*, *Sal*I; *Ap*, ampicillin resistance gene; *Tc*, tetracycline resistance gene; *FPP*, farnesyl diphosphate synthetase.

TABLE I

Prenyltransferase activity in yeast strain JGY193 and transformants harboring YRp17, pARC01, and pARC02

Strain	Time	Specific activity
	h	*nmol min*$^{-1}$ *mg*$^{-1}$
JGY193	13.5	5.7
	19.5	6.4
	24	4.9
	38	7.9
JGY193/YRp17	13.5	9.1
	19.5	6.4
	24	5.7
	38	7.1
JGY193/pARC01	13.5	18.0
	19.5	14.0
	24	14.0
	38	14.0
JGY193/pARC02	13.5	14.0
	19.5	12.0
	24	12.0
	38	11.0

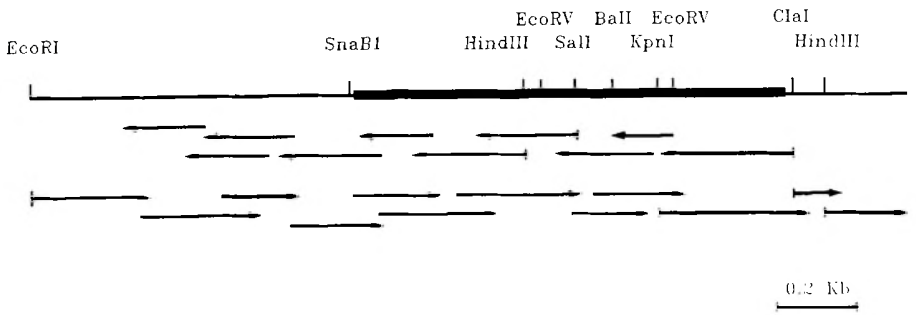


FIG. 2. **Strategy used to sequence the yeast FPP synthetase gene.** The *heavy bar* indicates the coding region of the gene. The direction of transcription is from left to right. The DNA sequence in the vicinity of the NH_2 terminus of the protein, and the direction of the gene was determined with pMA1A and pMA1B as template for the 33-mer used to identify the gene. Sequenced regions represented by *plain arrows* were propagated with synthetic 18-mers designed to hybridize to the ends of previously sequenced DNA. Sequences with *arrows containing vertical bars* were determined using a primer for the T7 region of the host plasmid. In these cases the region between the indicated restriction site in the insert and the corresponding site in pBluescript was deleted before sequencing. Overlapping sequences from left to right were obtained with pMA1A and from right to left with pMA1B. Restriction sites are as marked.

highly expressed gene in yeast (Sharp *et al.*, 1986). The probe was used to analyze Southern blots of separate genomic restriction digests (data not shown). The restriction digests of all 10 enzymes used contained a single DNA band which hybridized to the probe. Of these, five were less than 12 kb in length. Southern blots of yeast genomic DNA digested with pairs of these enzymes also showed binding to a single restriction fragment. Although the fragment sizes from two separate experiments showed some variation, a relatively consistent, partial restriction map of a unique genomic region binding this probe could be deduced.

A 4.5-kb *Eco*RI fragment was cloned first and, as shown below, was found to encode all information necessary for expression of the gene for FPP synthetase. DNA from a large scale *Eco*RI genomic restriction digest was separated on a preparative agarose gel, and the region containing DNA fragments which bound to the probe was retrieved. This set of fragments was ligated into pBR322, previously linearized with *Eco*RI, to create a selective library of genomic fragments. The library was then screened by colony lift hybridizations for those members harboring the fragment of interest. A single clone, designated pARC00, was found which contained a 4.5-kb *Eco*RI insert as shown in Fig. 1.

The direction of transcription of the putative gene for FPP synthetase was deduced by subcloning a 1.3-kb *Eco*RI-*Sal*I restriction fragment from pARC00 into M13mp18 and M13mp19. The single-stranded DNAs recovered from these clones were used as templates for sequencing reactions employing the 33-mer probe as a primer. The M13mp19-derived clone served as a template, whereas the M13mp18-derived clone did not. Thus, the direction of transcription is toward the interior of this insert as shown in Fig. 1.

Authenticity and Extents of the Putative Prenyltransferase Clone—In order to prove the existence of a functional structural gene for FPP synthetase in plasmid pARC00, we moved the 4.5-kb *Eco*RI insert into the yeast shuttle vector YRp17 (see Fig. 1). The two possible orientations of the insert, designated pARC01 and pARC02, were retrieved and separately transformed into *S. cerevisiae* strain JGY193. Transformants containing the putative gene for FPP synthetase, JGY193/pARC01, and JGY193/pARC02 along with the untransformed strain and a transformant containing the original shuttle vector JGY193/YRp17, were grown on selective media and assayed for their respective FPP synthetase activities. The results are summarized in Table I. The specific activity for FPP synthetase in the controls ranged from 4.9 to 9.1 nmol mg^{-1} min^{-1} while that of transformants containing pARC01 and pARC02 was 2–3-fold higher. Furthermore, strains transformed with pARC01 and pARC02 expressed equal amounts of prenyltransferase activity, indicating the presence of a functional promotor on the insert. The specific activity observed was relatively constant at all stages of growth tested.

Sequence of Prenyltransferase Gene—The 4.5-kb *Eco*RI insert of pARC00 was moved into the phagemid pBluescript SK(+), and clones containing both orientations of the insert, designated pMA1A and pMA1B, were isolated (see Fig. 1). The strategy used in sequencing the prenyltransferase gene, as well as the restriction map derived from the sequence data, are outlined in Fig. 2. Both strands were sequenced with overlaps to resolve any ambiguities. Restriction sites shown in Fig. 2 were verified by digestion of pARC00 with the appropriate enzymes.

The nucleotide sequence of the FPP synthetase gene and flanking DNA is shown in Fig. 3. An open reading frame was discovered which encoded a protein of 352 amino acids with a calculated molecular mass of 40,483 daltons. This value is in excellent agreement with the molecular mass of 40,000 daltons estimated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis of the purified protein blocked at the amino terminus and is within experimental error (±10%) of the 43,000-dalton molecular mass reported by Eberhardt and Rilling (1975). Although there are two potential glycosylation sites at asparagine 24 and asparagine 68, the almost identical molecular weights obtained by electrophoresis and from the gene sequence indicate either a lack of glycosylation or a low molecular weight species at these sites. The predicted pI of the protein is pH 5.1 and is in reasonable agreement with the value of 5.3 reported by Eberhardt and Rilling (1975).

Fig. 4*A* illustrates the similarity between the protein sequence derived from the baker's yeast enzyme and the corresponding sequence derived from the clone. The first amino acid determined by Edman degradation was from cycle 8, which corresponds to amino acid 9 of the cloned sequence. Although these amino acids do not match, the remaining Edman-derived sequence matches that of the clone exactly. The discrepancy could reflect a strain difference or merely an error in protein sequencing. The misalignment between these sequences by one amino acid suggests that except for the NH_2 terminal methionine, the amino terminus of the protein submitted for sequencing was intact. Fig. 4*B* shows the similarity between probe and clone sequences. Although there is a 21%

FIG. 3. **Nucleotide and presumptive amino acid sequence of the yeast FPP synthetase gene.** A poly(dA·dT) region and a potential TATA box and termination signals are *underlined.*

mismatch between the probe and the clone, these errors occur at the ends of the probe and apparently do not adversely affect hybridization. The significant identity of the clone sequence and probe codon choice is a reflection of the significant codon bias exhibited by this gene (see "Discussion").

In Fig. 5 the protein sequence of FPP synthetase is compared with that of the putative rat liver enzyme published by Clark *et al.* (1987). Also shown is a peptide fragment obtained from chicken liver FPP synthetase by Brems *et al.* (1981) using an active site-directed photoaffinity label (Brems and Rilling, 1979). Visual inspection alone reveals strong similarity throughout much of the sequences. Further comparison of FPP synthetase from our clone with the rat liver enzyme using the Dayhoff MDM-78 matrix method (Doolittle, 1981;

Needleman and Wunsch, 1970) revealed a highly significant alignment score of 32.3, where a score of three or greater indicates significant similarity.

Prenyltransferase Is an Essential, Single Copy Gene—A disruption-deletion mutation was made by fragment-mediated transformation (Rothstein, 1983) to determine whether the FPP synthetase gene was an essential, single copy gene in *S. cerevisiae*. Plasmid pARC00 was digested with *Sal*I to remove a 1.4-kb segment of the coding region of FPP synthetase. The *Sal*I fragment was replaced with a 2.2-kb *Xho*I-*Sal*I fragment from YEp13 containing the yeast *Leu2* gene. The resulting construct was digested with *Sna*BI and *Neo*I to yield a DNA fragment containing the *Leu2* gene flanked by sequences for FPP synthetase. This DNA fragment was used to transform the *leu2/leu2* diploid yeast strain JGY202 to *Leu2*$^+$. One chromosomal copy now contained the *Leu2*::FPP synthetase gene replacement, while the other chromosome contained the wild-type copy of the FPP synthetase gene. The chromosomal location of the insert was confirmed by the Southern blots shown in Fig. 6 of an *Eco*RI digest of genomic DNA from the transformed diploid using the 4.5-kb *Eco*RI fragment from pARC00 containing the FPP synthetase gene and the 2.2-kb *Xho*I-*Sal*I fragment from YEp13 containing the *Leu2* gene as ^{32}P-labeled probes. Chromosomal DNA from the parental strain gave single bands at 8 and 4.5 kb for the *Leu2* and FPP synthetase probes, respectively, while the deletion-disruption diploid showed an additional band near 2.6–2.7 kb consistent with the construction shown in *part A* of Fig. 6. The transformant was sporulated, and the phenotype of the null deletion mutant was analyzed by dissection of the resulting tetrads. In eight tetrads, two spores/tetrad were inviable and two were viable, both of which were leucine auxotrophs. The segregation of *Leu2* with the lethal mutation indicates that inactivation of the FPP synthetase gene is lethal in haploid cells.

A PROTEIN SEQUENCES:

From the purified protein:

cycle 8
N-Leu-**Glu-Arg-Phe-Leu-Asn-Val-Phe-Pro-Lys-Leu**-C

From the clone sequence:

amino acid 9
N-Arg-**Glu-Arg-Phe-Leu-Asn-Val-Phe-Pro-Lys-Leu**-C

B DNA SEQUENCES:

Probe sequence:

5′-TTG**GAAAGATTCTTGAACGTTTTCCGAAAGTTG**-3′

Clone Sequence:

5′-AGA**GAGAGATTCTTGAACGTTTTCCGTAAATTA**-3′

FIG. 4. **Comparison of protein and DNA sequences derived from purified yeast FPP synthetase and the sequenced gene.** *Part A*, the sequence from the purified protein was determined by Edman degradation, while that of the clone is presumptive. *Part B*, the probe is single-stranded DNA, constructed according to the NH_2-terminal sequence of FPP synthetase as described under "Experimental Procedures." The clone sequence is the presumed binding site of the probe. Identities between compared sequences are emphasized by *bold underlined letters.*

Prenyltransferase Resides on Yeast Chromosome XI—The chromosomal location of the gene for FPP synthetase was determined by using the entire 4.5-kb *Eco*RI insert from pARC00 as a probe against a Southern blot of separated yeast chromosomes. Although not all 17 yeast chromosomes are separated in this system, the resulting hybridization signal was unambiguous in this case and corresponded to chromosome XI as shown in Fig. 7. Further genetic analysis will be necessary to map the gene to a specific region of this chromosome.

DISCUSSION

In 1987, Clarke *et al.* reported the isolation from rat liver of a putative structural gene for FPP synthetase. Their study based this identification on the coordinate transcriptional regulation of the gene with that of hydroxymethylglutaryl-coenzyme A reductase, a critical step in the initiation of isoprene biosynthesis, and they found a striking resemblance between the central portion of their clone's inferred amino acid sequence and a cyanogen bromide-derived fragment isolated by Brems *et al.* (1981) from purified chicken liver FPP synthetase. This fragment, identified through the use of an active site-directed photoaffinity label, is believed to be a component of the allylic binding site (Brems *et al.*, 1981). More recently, Ashby and Edwards (1989) identified the function of the rat liver clone by constructing a fusion protein containing the amino terminus of bacterial anthranilate synthetase and the full length rat liver polypeptide. Antisera directed against the fusion protein inactivated prenyltransferase activity in a cell-free rat liver preparation. They also found that affinity purified IgG to purified avian liver farnesyl diphosphate synthetase cross-reacted with the fusion protein.

We isolated the structural gene encoding yeast FPP synthetase from genomic DNA by hybridization of a synthetic oligonucleotide probe based on the NH_2-terminal sequence of the enzyme. The clone, confirmed by expression of prenyltransferase activity, encodes a protein which shows substantial sequence similarity to the rat liver clone of Clarke *et al.* (1987) and confirms their assignment. As shown in Fig. 5,

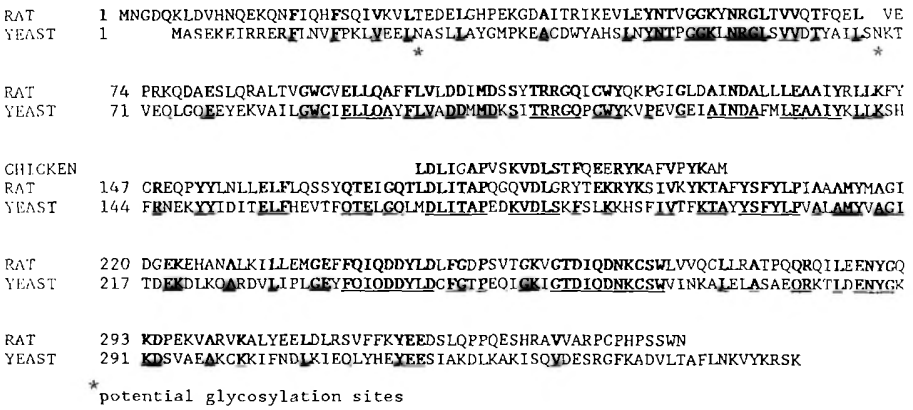


FIG. 5. **Comparisons among the yeast and rat liver FPP synthetase and the active site fragment of the chicken liver enzyme.** The sequence of the rat liver protein is taken from Clarke *et al.* (1987). That for the chicken liver fragment is from Brems *et al.* (1981). The first 71 amino acids of the rat liver enzyme are offset by two relative to the remainder of the sequence for the yeast protein in order to maximize homology at the amino terminus. Identical residues are *bold and underlined. Asterisks* by the yeast sequence indicate potential glycosylation sites.

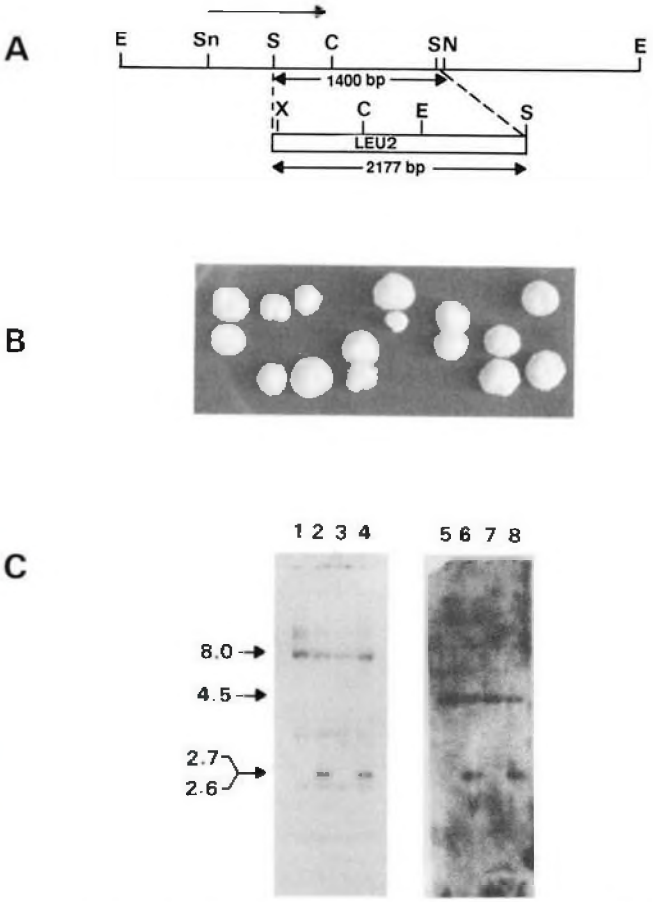


FIG. 6. **Deletion-disruption of the FPP synthetase gene.** *Part A*, a graphic description of the deletion-disruption FPP synthetase mutant. A 1400-bp *Sal*I fragment, which included the coding region for amino acids 180–352 in FPP synthetase in pARC00, was replaced by a 2177-base pair DNA fragment containing the yeast *leu2* gene. The direction of FPP translation is indicated by the *arrow*. Restriction sites are *E* = *Eco*RI, *Sn* = *Sna*BI, *S* = *Sal*I, *C* = *Cla*I, *X* = *Xho*I, *N* = *Nco*I. *Part B*, tetrad analysis of a *Leu*$^+$ diploid strain obtained by transformation with a *Sna*BI-*Neo*I fragment from the deletion-disruption construction described in *part A*. After the transformant was sporulated, asci were dissected and gave the indicated patterns. The haploid yeast shown were all leucine auxotrophs. *Part C*, Southern analysis of DNA isolated from the nondisrupted diploid strain (*lanes 1, 3, 5,* and *7*) and from the deletion-disruption diploid strain (*lanes 2, 4, 6,* and *8*). Five μg of yeast DNA from the indicated strains were digested with *Eco*RI, electrophoresed, and transferred to nitrocellulose paper as described under "Experimental Procedures." *Lanes 1–4* were probed with a nick-translated *Leu2* probe, while *lanes 5–8* were probed with the 4.5-kb FPP gene fragment.

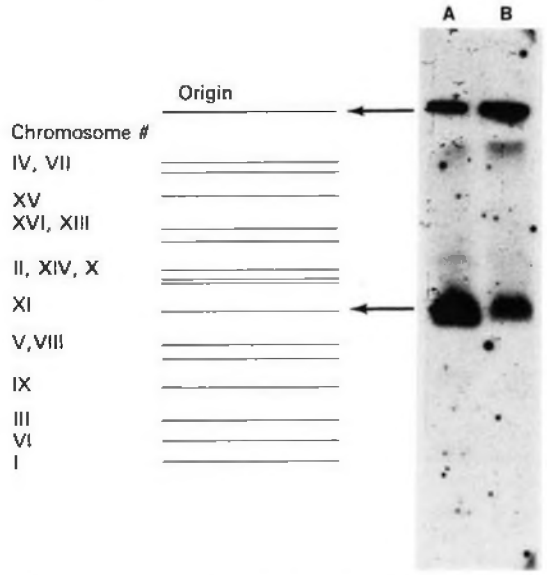


FIG. 7. **Chromosomal location of the FPP synthetase gene.** An autoradiogram of a yeast chromosomal Southern blot, probed with a nick-translated 4.5-kb *Eco*RI restriction fragment containing the yeast FPP synthetase gene shows a single band at a location unique to chromosome XI. *Lanes A* and *B* are duplicate runs of *S. cerevisiae* chromosomes separated into 16 distinct bands by CHEF gel electrophoresis.

44% of the amino acids are identical, and this value increases to 66% when conservative substitutions are considered. Sheares[2] has recently used cDNA from the rat liver gene to probe a human cDNA library and found a putative clone for a human gene which encodes a protein with high similarity to the rat liver and yeast enzymes.

In all of our Southern blot experiments with restricted yeast genomic DNA hybridized to a single-stranded DNA probe, we consistently identified a single radioactive band, unique in length to the restriction enzyme used. The fragments thus identified were consistent with a single genomic locus for the FPP synthetase gene, which we designate as *bot3*. This suggestion is supported by the observation that insertional inactivation of the FPP synthetase gene in haploid yeast cells is lethal, indicating the loss of an essential, single copy gene. In contrast, the rat liver prenyltransferase gene exists in at least five genomic copies (Clarke *et al.*, 1987).

Visual inspection of the yeast FPP synthetase DNA sequence reveals several potential regulatory sites. A consensus TATA box, spaced 125 nucleotides from the presumed initiating methionine, is located at position 666 of the DNA sequence. This is within the spacing window of TATA boxes found in other yeast genes (Hahn *et al.*, 1985, Nagawa and Fink, 1985). At the end of the coding sequence are several elements described by Zaret and Sherman (1982), indicated by *underlines* in Fig. 3, that could possibly be involved in transcription termination.

In *S. cerevisiae* sterols typically constitute 1.2–3.2% of the dry weight of the cell (Longley *et al.*, 1968; Hunter and Rose, 1972), and yeast is a good source for many of the enzymes involved in cholesterolgenesis. The specific activity of FPP synthetase in disrupted yeast (see Table I) is similar to that found in animal tissues such as liver, which serves as a major site of cholesterol biosynthesis (Reed and Rilling, 1975). The level of transcription of the FPP synthetase gene in yeast can be estimated by the codon bias exhibited in the reading frame (see Table II). Bennetzen and Hall (1982) have shown that the more abundant an mRNA species in a yeast cell, the more biased is this mRNA toward the use of a 22 codon subset of the 61 codon genetic dictionary. These workers devised the codon bias index (CBI) as a measure of this discrimination, wherein a value of 0.0 indicates totally random codon usage, a value of 1.0 indicates exclusive use of the 22 codon subset, and intermediate values reflect the percentage bias. More recently, Kammerer *et al.* (1984) and Sharp *et al.* (1986) have expanded the number of similarly indexed yeast proteins to over 100, providing a data base from which comparisons may be made. FPP synthetase with a CBI of 0.52 is more biased than most genes encoding biosynthetic enzymes (CBI approximately 0.1–0.6), comparable to the *leu2* gene (CBI = 0.50) and ubiquitin (CBI = 0.50), but lower than structural genes such as those encoding histones (CBI approximately 0.7–0.8)

[2] B. Sheares, Merck Sharp & Dohme, personal communication.

TABLE II
Codon usage table

AA, amino acid.

AA	Codon	No.	AA	Codon	No.	AA	Codon	No.	AA	Codon	No.
Phe	TTT	0	Ser	TCT	6	Tyr	TAT	4	Cys	TGT	3
	TTC	16		TCC	6		TAC	16		TGC	3
	TTA	6		TCA	3		TAA	0		TGA	0
	TTG	23		TCG	1		TAG	1		TGG	4
Leu	CTT	3	Pro	CCT	5	His	CAT	1	Arg	CGT	1
	CTC	1		CCC	0		CAC	4		CGC	0
	CTA	4		CCA	4	Gln	CAA	10		CGA	0
	CTG	0		CCG	0		CAG	4		CGG	0
Ile	ATT	12	Thr	ACT	6	Asn	AAT	4	Ser	AGT	1
	ATC	9		ACC	6		AAC	9		AGC	1
	ATA	2		ACA	1	Lys	AAA	14	Arg	AGA	9
Met	ATG	7		ACG	2		AAG	21		AGG	1
Val	GTT	9	Ala	GCT	9	Asp	GAT	12	Gly	GGT	10
	GTC	8		GCC	10		GAC	12		GGC	4
	GTA	2		GCA	8	Glu	GAA	24		GGA	0
	GTG	1		GCG	1		GAG	7		GGG	2

and ribosomal proteins (CBI approximately 0.8–0.95) (Sharp *et al.*, 1986). We estimate from our purification that FPP synthetase constitutes approximately 0.1% of the cytosolic protein in yeast. In comparison, the gene encoding isomerase has a CBI of only 0.24 (Anderson *et al.*, 1989). Furthermore, FPP synthetase activity is consistently present at three to four times the level of isopentenyl diphosphate isomerase in crude yeast extracts.[3]

Bernard and Popjak (1980) suggested that two arginyl residues in porcine liver FPP synthetase may be involved in the binding of substrates. Further, Brems *et al.* (1981) found an arginine residue was the most heavily radiolabeled amino acid in a polypeptide fragment isolated from avian liver FPP synthetase by an active site-directed photoaffinity probe. Yeast FPP synthetase encodes a lysine residue at this position (Lys-189), thereby maintaining a positive charge at this position, but with different geometric constraints. The notion that charge at this locus may be necessary for interaction with the pyrophosphate moiety of an allylic substrate (Brems *et al.*, 1981) may now be tested by site-directed mutagenesis.

FPP synthetase is but one of a family of prenyltransferases found in a typical cell. Individual members generally exhibit a high degree of specificity for the allylic diphosphates and acceptors they utilize as substrates. However, the same allylic diphosphate (DMAPP in FPP synthetase and DMAPP-tRNA transferase) or acceptor (IPP in FPP synthetase, undecaprenyl-PP synthetase, and dolichyl-PP synthetase) may serve as a substrate for different prenyltransferases that occur in the same organism. In this regard, it is interesting to note that the 4.5-kb *Eco*RI DNA fragment from pARC00 containing the gene for FPP synthetase hybridized cleanly to a single chromosome. Furthermore, yeast FPP synthetase showed no significant similarity with the yeast *MOD5* gene product, a tRNA modifying prenyltransferase that utilizes DMAPP to alkylate the amino moiety of A37 in tRNAs which read codons beginning with U (Najarian *et al.*, 1987). Likewise, no similarity is seen for the prenyltransferase from *P. savastanoi* (Powell and Morris, 1986) or the closely related enzymes encoded by the *tzs* or *tmr* genes in *A. tumefaciens* (Akiyoshi *et al.*, 1985; Barry *et al.*, 1984) which catalyze alkylation of the amino group in AMP by DMAPP. There was also no significant similarity between yeast isomerase (Anderson *et al.*, 1989) and yeast FPP synthetase, although both utilize IPP and DMAPP as substrates. Thus, there do not appear to be highly conserved protein sequences for binding IPP or allylic diphosphates in the different isoprenoid enzymes studied to date which utilize these substrates.

[3] M. Muehlbacher and C. D. Poulter, unpublished observations.

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Note Added in Proof—A comparison of DNA sequences shows that the unidentified open reading frame designated URF$_x$ by Maarse and Grivell (Maarse, A. C., and Grivell, L. A. (1987) *Eur. J. Biochem.* **165,** 419–425) encodes the C-terminal fragment of FPP synthetase beginning with Met-131. Thus, bot3 is located immediately upstream from the gene for the 11-kDa subunit VIII of ubiquinol-cytochrome-*c* oxidoreductase. It is also interesting to note that cyanogen bromide fragments 3, 4, and 8 of *cis*-polyisoprene rubber transferase from *Hevea brasiliensis* have significant similarity to sequences Ile-307-Lys-320, Gly-111-Lys-117, and Lys-148-Ile-173, respectively (Light, D. R., and Dennis, M. S. (1989) *J. Biol. Chem.* **264,** 18589–18597).

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